Aus dem Institut für Neuropathologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Impact of inflammasome inhibition on the development of insulin resistance in the context of diet-induced obesity

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

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Datum der Promotion:

25.11.2022

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Abbreviations

%	percentage
°C	degree Celsius
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	adenosine triphosphate
AUC	area under the curve
BAT	brown adipose tissue
BCA	bicinchoninic acid
Bl6	C57 black 6
BMI	body mass index
BrdU	bromodeoxyuridine
BW	bodyweight
C	control
CARD	caspase recruitment domain-containing protein
CD11b	cluster of differentiation molecule 11b
CD68	cluster of differentiation molecule 68
CHOW	control chow
CHOW-C	control chow-fed mice treated with vehicle
CHOW-I	control chow-fed mice treated with Ebselen
CNS	central nervous system
CO2	carbon dioxide
CRP	C-reactive protein
CSF-1	colony-stimulating factor
CVD	cardiovascular disease
d	day
DAB	diaminobenzidine
DAMPS	damage-associated molecular pattern
Db/db mouse	leptin receptor deficient mouse, model for T2D
ddH2O	distilled deionized water
DIO	diet-induced obesity
Dl	deciliter
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
e.g.	exempli gratia, for example
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
G	gauge
g	gram
h	hour
H2O	water
H2O2	hydrogen peroxide
HBSS	Hanks balanced salt solution
HCl	hydrochloric acid
HFD	high-fat diet
HFD-C	high-fat diet-fed mice treated with vehicle
HFD-I	high-fat diet-fed mice treated with Ebselen
HFD-IO	high-fat diet-induced obesity
H-NMR	nuclear magnet resonance spectroscopy
I	inhibition (Ebselen)
i.p.	intraperitoneal
IL	Interleukin
IO	induced obesity
ipGTT	intraperitoneal glucose tolerance test
kcal	kilocalories
kg	kilogram
LPS	lipopolysaccharide
M	molar
MACS	magnetic-activated cell separation
mg	milligram
min	minute
mRNA	messenger RNA
MSD	Meso Scale Discovery
NaCl	sodium chloride
NALP3	NACHT, LRR and PYD domains-containing protein 3 = NLRP3
NaOH	sodium hydroxide
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NGS	normal goat serum
NLR	Nod-like receptor

NLRP3	NLR family pyrin domain containing 3
NPY	neuropeptide Y
O2	oxygen
PAMPS	pattern-associated molecular pattern
PBS	phosphate buffered saline
PBS-TX	PBS with Triton-X100
PCR	polymerase chain reaction
PenStrep	Penicillin Streptavidin
PFA	paraformaldehyde
Pg	picogram
Pm	peritoneal macrophages
PRR	Pattern recognition receptor
PYCARD	ASC
qRT-PCR	quantitative real time PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcription PCR
s	second
SAT	subcutaneous adipose tissue
SD	standard deviation
SDS	rodium dodecyl sulfate
T2D	type 2 diabetes mellitus
TBS	tris buffered saline
TEE	total energy expenditure
Th	T Helper
TLR	Toll-like receptor
TMC	total movement counts per day
Tris	tris-(hydroxymethyl)-aminomethane
US	United States
UV	ultraviolet
V	volume
VAT	visceral adipose tissue
WAT	white adipose tissue
WT	wild type
Mg	microgram

1 Abstract

High-fat diets (HFD) cause inflammation both in the periphery and the central nervous system. In adipose tissue, inflammation is mainly mediated by macrophages that are recruited to the tissue. Similarly, microglia, the innate immune cells of the brain, are activated in the hypothalamus of mice fed an HFD. Several studies have shown that the nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain containing 3 (NLRP3) inflammasome, which is expressed by microglia and macrophages, plays a pivotal role in the metainflammation caused by diet-induced obesity (DIO). We were able to use a potential pharmacological NLRP3 inflammasome inhibitor called Ebselen to test the hypothesis that a selective pharmacological inhibition of the NLRP3 inflammasome reduces HFD-induced metabolic alterations. First, we showed that Ebselen was able to inhibit IL-1β secretion in peritoneal macrophages and microglia. We also tested the effects of Ebselen *in vivo* using male C57/BL 6 wild-type mice fed on a control diet or a high-fat diet, as well as a subgroup of HFD mice and CHOW mice treated with the potential NLRP3 inflammasome inhibitor Ebselen (intraperitoneal injections of 10 mg/kg 3 times a week) or a vehicle. HFD feeding increased weight and impaired glucose homeostasis. Intraperitoneal Ebselen treatment averted weight gain and insulin resistance in HFD-IO (HFD-induced obesity) mice with no change in caloric intake. This effect may be due to the inhibition of IL-β secretion of the NLRP3 inflammasome; however, we cannot exclude that these effects might have been a consequence of drug toxicity, as the mice treated with Ebselen exhibited peritonitis and a high mortality rate. To avoid the side effects of the intraperitoneal injections of Ebselen, we changed the method of administration. The mice were given the same dose of Ebselen as before, but this time it was administered through their drinking water. In this experiment, Ebselen did not affect the w

eight gain in HFD mice, but the drug prevented the development of insulin resistance in HFD-IO mice after 1 month of treatment. After 2 months of treatment, this effect had vanished. After 10 weeks of treatment, HFD-IO mice did not exhibit elevated IL-1β levels, so the long-term effects of Ebselen on the IL-β level could not be assessed. In addition to proving that Ebselen can inhibit the NLRP3 inflammasome, further research into alternative application routes and drug formulations must be carried out before Ebselen can become a viable therapeutic option.

2 Zusammenfassung

Stark fetthaltige Nahrung (HFD) verursacht eine Entzündung in der Peripherie sowie in dem zentralen Nervensystem. Im Fettgewebe wird die Entzündung hauptsächlich durch Makrophagen ausgelöst. Analog wurde gezeigt, dass HFD und Übergewicht eine Aktivierung von Mikroglia, den angeborenen Immunzellen des Gehirns, im Hypothalamus von Mäusen auslöst. Mehrere Studien zeigten, dass NLR family, pyrin domain containing 3 (NLRP3) Inflammasomes eine entscheidende Rolle in dieser peripheren sowie zentralen Meta-Inflammation in der Pathophysiologie von HFD und Übergewicht hat. Unser Labor hat die bis jetzt unveröffentlichte Information erhalten, dass das Pharmakologen Ebselen das NLRP3 Inflammasome pharmazeutisch hemmt. Daher testeten wir die Hypothese, ob Ebselen, durch eine selektive Hemmung des NLRP3 Inflammasomes zu einer Reduktion der metabolischen Veränderungen bei durch fettreiche Ernährung ausgelöstem Übergewicht führt. Zuerst zeigten wir, dass Ebselen die IL-1β-Sekretion in peritonealen Makrophagen und Mikroglia *in vitro* hemmt. Für die *in vivo*-Experimente wurden männliche C57 /BL6- Wildtyp-Mäuse mit entweder fettreicher Nahrung oder Kontrollfutter gefüttert. Diese beiden Gruppen wurden dann jeweils in zwei Gruppen, die entweder mit dem potentiellen NLRP3 Inflammasome Inhibitor Ebselen (10mg/kg dreimal pro Woche intraperitoneal) oder als Kontrolle nur mit dem Lösungsmittel behandelt. In der Kontrollgruppe, die mit HFD gefüttert wird, entwickelten die Mäuse Übergewicht und eine beeinträchtige Glukosehomöostase. Die intraperitoneale Injektion mit Ebselen führte in der HFD und in der Kontrollfutter Gruppe zu einer Peritonitis bis hin zum Tod. Daher ist die reduzierte Gewichtszunahme und verbesserte Glukosehomöostase in der mit Ebselen behandelten HFD Gruppe, wahrscheinlich auf die Erkrankung der Mäuse zurück zu führen. Die Menge der aufgenommenen Kalorien in beiden HFD Gruppen war identisch. Ob die Gewichtsreduktion und verbesserte Insulinsensitivität auch auf der pharmakologischen Hemmung des NLRP3 Inflammasomes beruht, können wir nicht ausschließen. Um die Nebenwirkungen der intraperitonealen Injektion zu vermeiden, wurde als alternativer Applikationsweg Ebselen in Trinkwasser aufgelöst und den Mäusen darüber zugeführt. In diesem Experiment hatte Ebselen keinen Einfluss auf die Gewichtszunahme. Nach einem Monat Behandlung verhinderte Ebselen die Entwicklung einer Insulinresistenz. Dieser Effekt war jedoch zu einem späteren Zeitpunkt verschwunden. Nach 10-wöchiger Behandlung hatte keine der HFD Gruppen erhöhte IL-1β Spiegel in weißem Fettgewebe, so dass die Wirkung von Ebselen auf den IL-β Spiegel nicht untersucht werden konnte. Neben der Veröffentlichung der Hemmung der NLRP3 Inflammasomes durch Ebselen sind weitere Untersuchungen zu

alternativen Anwendungswegen und Arzeimittelrezeptur notwendig, um Ebselen zu einer Behandlungsmöglichkeit bei Übergewicht zu etablieren.

3 Introduction

3.1 Obesity und metabolic diseases

Obesity, which is defined by a Body Mass Index (BMI=body weight in kg divided by height in m²) of over 30 kg/m^2, is one of today's major health problems. According to the WHO, 39% of adults aged 18 years and over were overweight in 2016 (BMI ≥ 25 kg/m²), and 13% of these were even considered obese (BMI ≥ 30 kg/m²). To underline the severity of obesity, which reduces life expectancy by an average of 6 years [2], obesity was defined as an illness by the American Medical Association in 2013, at the same time recognizing that obesity is not only the result of an unhealthy lifestyle. The prevalence of obesity has risen dramatically in recent years. As a result, more people suffer from obesity-related chronic metabolic and inflammatory multiorgan disorders (e.g., pancreatic, adipose, hepatic, cardiac and muscle tissue), known as the "metabolic syndrome". The syndrome includes impaired insulin sensitivity, pancreatic ß-cell dysfunction, dyslipidemia, atherosclerosis, hypertension and fatty liver disease.

The causes of obesity are manifold and not yet fully understood. Monogenic or syndromic obesity account for only about 5% of obesity in humans, although twin studies suggest that genetic influence has a major impact [3, 4]. The reason for the rapid increase in obesity is thought to be changes in the environment and lifestyle.

Obesity is caused by an imbalance between energy intake and energy expenditure. Excess energy may result from a changed diet, especially in western countries. Larger portions, higher caloric density and easier access to food contribute to this [5]. Along the same lines, epidemiological studies reveal a positive correlation between increased fat content in the diet and an increased incidence of obesity [6]. Furthermore, there is an inverse correlation between adiposity and physical activity [7]. The WHO states that 30% of the world's population gets insufficient exercise [8]. Finally, social factors play an important role, since people whose friends become obese are 57% more likely to become obese themselves [9].

In addition to the problem of the increasing incidence of obesity, there is little or no evidence that a change of lifestyle can successfully treat the illness, especially in the long term [10, 11]. In the long term, only 2-20% of people succeed in reducing their weight [12]. It is therefore predicted that by 2035, obesity will account for 20% of the total healthcare costs in western countries.

Diseases associated with obesity can be roughly subdivided into two groups. The first group is caused mainly by increased weight and fat mass, such as sleep apnea and degenerative diseases of the musculoskeletal system, including arthrosis. The second group of diseases arises from metabolic disorders and includes diabetes mellitus type 2 (T2DM).

Nearly 10% of adults worldwide suffer from T2D, which causes 5% of deaths secondary to comorbidities including cardiovascular disease (CVD), neuropathy and nephropathy [13]. Lifestyle interventions in obesity have a minimal effect on CVD mortality in obese patients [14].

As a result of the great prevalence and morbidity of obesity and T2D, efforts must be made to find new innovative pharmacological approaches for the treatment and prevention of obesity and its complications, such as T2D. This treatment could be an alternative for people who are unable to reduce their bodyweight and become metabolically healthy using classic therapeutic principles.

3.2 Obesity-induced inflammation

Following the landmark discovery that adipose tissue of obese animals produces pro-inflammatory cytokines [15, 16], the link between inflammation in metabolically active organs and metabolic diseases, including diet-induced obesity (DIO) and insulin resistance, leading to T2D, has been substantiated by intense research efforts [17]. Inflammation caused by obesity has been observed in other metabolically active organs, such as the liver and pancreas [18-20]. This chronic low-grade inflammation is defined as metabolic inflammation (i.e., meta-inflammation) [21].

Weight gain causes expansion and hypertrophy in adipose tissue (AT). When the storage capacity of the adipocytes is exceeded, fatty acids are accumulated in non-AT, such as the liver, skeletal muscle and pancreas, and are found in the blood stream as free fatty acids, causing the activation of inflammatory pathways and lipotoxicity [22]. At this point, immune cells infiltrate diverse tissues, including AT, and are activated. These pathways result in cellular dysfunction [23], ultimately promoting metabolic disorders, such as insulin resistance. In the years following these discoveries, macrophages have been identified as the major source of obesity-associated tissue inflammation [24, 25].

Analogously to inflammation in peripheral organs, increased inflammation in the central nervous system (CNS) has also been reported and found to be associated with diet-induced weight gain [26, 27]. As in the periphery, the inflammatory response in the CNS is primarily characterized by activation of microglia, the intrinsic macrophages of the brain. The hypothalamus controls appetite and energy expenditure by responding to metabolic endocrine signals and affecting insulin release from the pancreas and leptin from adipose tissue [28]. Obesity has long been associated with insulin or leptin resistance in peripheral tissues, but recently it has been shown that the hypothalamus also develops resistance to these hormones [29–31], thus promoting the development of metabolic disorders.

In recent years, the exact mechanisms of the activation of the immune system in the context of obesity have been investigated, and sterile inflammation, which is defined as an inflammation caused without any pathogens, seems to be the key pathomechanism.

3.3 Sterile inflammation and the NLRP 3 inflammasome

Normally, inflammation is a protective immune response to harmful stimuli. As innate immune cells, macrophages and microglia express a variety of pattern recognition receptors (PRRs). Among the PRRs, pathogen-associated molecular pattern (PAMPS) receptors are used to detect exogenous pathogens, while danger-associated molecular pattern (DAMP) receptors allow the recognition of endogenous signals that may be present after tissue damage [29]. Examples of identified DAMPs include reactive oxygen species (ROS), adenosine triphosphate (ATP), hypotonic stress, uric acid crystals, asbestos, UV radiation, saturated fatty acids, ceramide, palmitate, amyloid beta and cholesterol crystals [30, 31]. DAMP-triggered inflammation is called sterile inflammation when it occurs in the absence of foreign pathogens, such as bacteria [32]. The activation of PRRs leads to the activation of downstream signaling cascades and triggers the production of interferon-α, interferon-β and other pro-inflammatory cytokines. Among the PRRs that bind endogenous danger signals are the NOD-like receptors (NLRs). The NLR family contains proteins that form large cytosolic protein complexes, called inflammasomes. The best characterized inflammasome is that formed around NLRP3/NALP3 [31].

The NLRP3 inflammasome is a huge cytosolic protein complex which assembles after sensing DAMPS or PAMPS and serves as a scaffold to recruit caspase-1 [33]. The NLRP3 inflammasome consists of an intracellular sensor, the precursor procaspase-1, and the

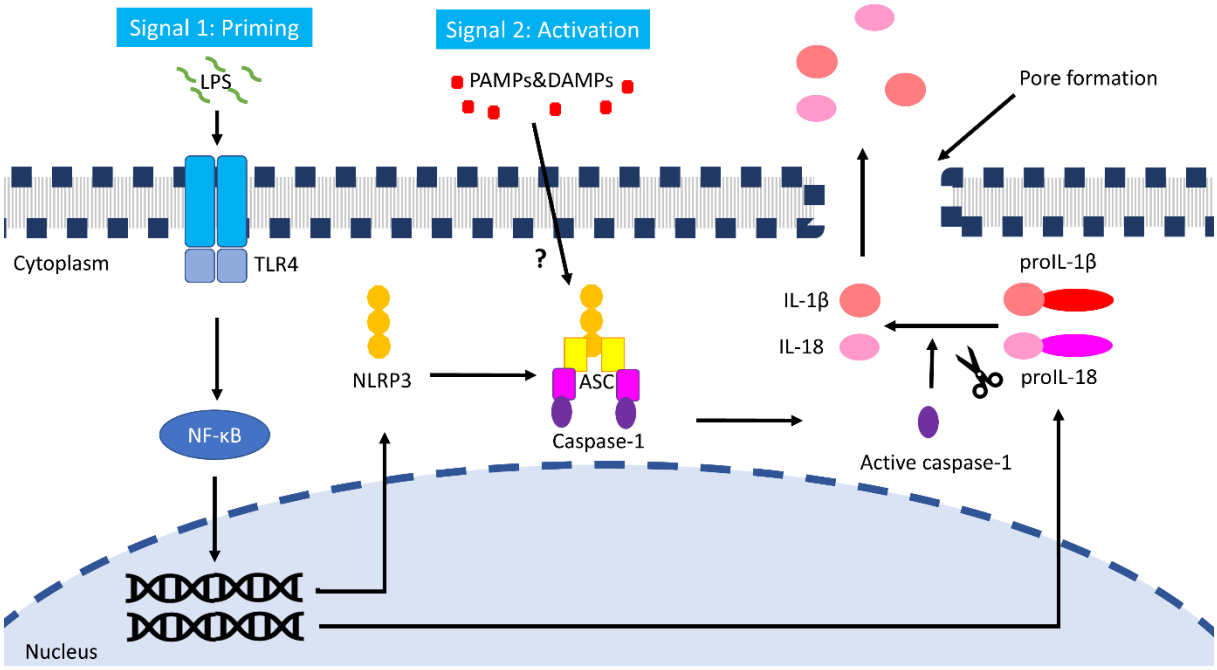

Figure 1: *Mechanism of the activation of the NLRP3 inflammasome: LPS induces TLR4 (Toll-like receptor 4)-dependent activation of NF-κB, resulting in NLRP3, proIL-1β and proIL-18 expression (**Signal 1/Priming**). NLRP3 inflammasome assembly (NLRP3, ASC and Caspase-1) is activated by DAMPs/PAMPs (**Signal 2/ Activation**). Caspase-1 cleaves the pro-inflammatory cytokines proIL-1β and -18. Pyroptosis, a lytic form of cell death, is also triggered by caspase-1, through pore formation. This simplifies the release of the active cytokines IL-1β and IL-18. IL-1β, as well as its pyrogenic characteristics, promotes adaptive T Helper 1 (Th1), Th17. IL-18 then triggers the IL-18 secretion in Th17 cells [1].*

apoptosis-associated speck-like adaptor protein containing a CARD (ASC) [33]. Activation via [metabolic] danger signals leads to the autocatalytic activation of the cysteine-dependent protease caspase-1 [34]. The resultant p10 and p20 caspase-1 subunits assemble to form active caspase 1, which acts to convert precursor cytokines pro-IL-1β and pro-IL-18 into their metabolically active forms [35]. Furthermore, caspase-1 leads to the formation of large pores resulting in a lytic mode of cell death, pyroptosis, which acts as a key defense against microbial infections [36].

Although the exact molecular pathways of the NLRP 3 inflammasome assembly are not completely known, it is agreed that NLRP3 activation is a two-step process requiring two different sequenced signals. The first signal is known as priming and activates the nuclear factor κB (NF-κB) through TLR ligation, which is required for cells to express pro-IL-1β and NLRP3 protein. Microbial molecules (e.g. bacterial lipopolysaccharide [LPS]) can act as the priming signal (**signal 1**) [37]. Therefore, most experimental *in vitro* protocols studying NLRP3 inflammasome activation include "priming" with a TLR agonist, such as LPS. After being primed, the NLRP3 can respond to its stimuli and can assemble the NLRP3 inflammasome. Following this, the priming of a second stimulus (**signal 2**), such as ATP, nigericin, or bacterial

toxins, is required for maximum IL-1β release and full inflammasome activation [38]. The exact mechanisms leading to NLRP3 inflammasome activation are the subject of debate **(Figure 1**).

3.4 NLRP3 in diabetes and obesity

In recent years, sterile inflammation through the activation of the NLRP3 inflammasome has been linked to a variety of autoimmune and autoinflammatory diseases, including neurodegenerative diseases (e.g. Alzheimer's disease) and metabolic disorders (obesity, type 2 diabetes and atherosclerosis) [39].

Particularly in HFD-IO obesity and the development of insulin resistance, the activation of the NLRP3 inflammasome seems to be the driving force. There is increased activation of the NLRP3 inflammasome and caspase-1 in the metabolic organs of obese mice and humans, whereby elevated levels of NLRP3 and ASC/PYCARD have been found in adipocytes of obese patients [40, 41]. Human studies have shown that augmented activity of the NLRP3 inflammasome and IL-1β in white adipose tissue distinguishes the metabolically unhealthy from the metabolically healthy [42]. Furthermore, there is a correlation between the concentration of the inflammasome components and the severity of type 2 diabetes [41, 43].

Animal studies have underlined the importance of the NLRP3 inflammasome in the development of obesity itself and of obesity-related diseases. Studies on animals lacking inflammasome components through genetic knockout in the context of HFD-induced obesity or genetically induced obesity have shown that NLRP3-, ASC- and caspase-1 deficiency protect against HFD-induced obesity [44]. Mice lacking Casp-1 had reduced adipocyte size and fat mass, and improved insulin sensitivity [41, 45].

Il-1β in particular, a product of the inflammasome, has been strongly linked to the development of type 2 diabetes, causing β-cell functional impairment and apoptosis. IL-1β weakens insulin sensitivity by inducing JNK-dependent serine phosphorylation of insulin receptor substrate, disrupting the PI3K-Akt-signaling in insulin-targeted cells [46]. There is a causality between IL-1β and the development of type 2 diabetes, since increased plasma levels of IL-1β and IL-6 augment the risk of T2D [47].

The pathomechanisms of the activation of the NLRP3 inflammasome in obesity are the subject of intense research. It has been shown that the NLRP3 inflammasome is triggered by saturated fatty acids, such as palmitate and ceramide. These saturated fatty acids are elevated in obesity,

thus linking the development of insulin resistance to obesity and aggravating obesity itself. Interestingly, omega-3 fatty acids inhibit NLRP3 inflammasome activity. Insulin resistance is further promoted by glucose, which has been shown to be able to activate the NLRP3 inflammasome.

3.5 Human studies and health implications

Consequently, one promising therapy for obesity and T2D, in addition to lifestyle intervention, would be the inhibition of the NLRP3 inflammasome. As described above, genetic elimination of the NLRP3 inflammasome in mice protects against metabolic diseases, including the development of insulin resistance. Given these ameliorative effects, a pharmaceutical NLRP3 antagonist would be of great importance.

Different therapeutic strategies to inhibit the NLRP3 inflammasome or its products have already been investigated. The most widely used inflammasome-related therapeutics are interleukin-1 receptor antagonists, such as Anakinra. Apart from inhibiting IL-β, IL-α is also affected by this drug, making it less specific. Furthermore, there are other aspects of Anakinra which make the antibody (AB) unsuitable for the long-term treatment of type 2 diabetes, such as it being very expensive as a monoclonal AB and the need for daily injections due to its short half-life.

Canakinumab is a human monoclonal antibody targeted at interleukin-1β, without cross-reactivity with IL-α. In a clinical study, Canakinumab did not reduce the incidence of new-onset diabetes, and although it reduced HbA1c during the first 6-9 months of the study, no long-term benefits were achieved [48]. Even though a reduction in deaths from cardiovascular disease was seen, there was no overall survival benefit and the drug caused numerous side-effects, making Canakinumab a less favorable drug in treating T2D and cardiovascular disease in obese patients. Neither Canakinumab nor Anakinra inhibit the NLRP3 molecule or complex, and they do not, therefore, inhibit IL-18 or caspase 1. Since pharmacological studies of both drugs did not show any great effect and considering the cost of the drugs, a specific NLRP3 inhibitor could be a better option.

At the start of our experiments, no other specific NLRP3 inflammasome inhibitor existed. We were therefore very excited when Veit Hornung, from the University of Bonn, informed us of a newly-discovered - and prior to this study unknown - property of Ebselen: the specific inhibition of the NLRP3 inflammasome.

3.6 Aims of this study

This recently identified safe and efficient inflammasome inhibitor, which was also assessed in *in vivo* studies (personal communication with Veit Hornung, University of Bonn), will be tested *in vitro* for efficacy (**Aim 1**) and *in vivo* to assess the effects of inflammasome inhibition on high-fat diet-induced metabolic syndrome in wild-type mice (**Aim 2**).

1. Primary peritoneal macrophages and microglia will be exposed to LPS and ATP, inducers of inflammasome activation, in the presence or absence of Ebselen, the supposed NLRP3 inflammasome inhibitor, to assess the efficacy of inflammasome inhibition in response to metabolic inflammasome activators. The medium will be tested for IL-1β.

2. Wild-type mice will be fed either a high-fat diet or standard chow control diet for 10 weeks while being treated with an NLRP3 inflammasome inhibitor or vehicle control treatment to determine whether inflammasome inhibition in the context of a high-fat diet is efficacious in ameliorating diet-induced metabolic syndrome.

The animals will be metabolically phenotyped throughout the experiment and at the end of the experiment to evaluate metabolic status (weight gain, energy expenditure, body mass, food intake, insulin sensitivity), and the IL-1β levels in the blood and white adipose tissue (WAT) will be measured.

4 Methods

4.1 *In vitro* experiments

4.1.1 Extraction and culturing of primary peritoneal macrophages

For the extraction of peritoneal macrophages, Bl6 mice were euthanized with dry CO2. Then the peritoneum was quickly and gently exposed and 10 ml ice cold PBS was slowly injected intraperitoneally with a 23G needle. Each mouse was shaken for one minute to harvest as many peritoneal macrophages as possible. The liquid was collected from the cavity using a 20G needle. An average of 8 ml was collected. The collected cells were centrifuged for 10 min at 500 rpm. They were then counted and plated with 500 000 cells per 24-well plate in Roswell Park Memorial Institute medium (RPMI; Invitrogen) supplemented with 10% FBS and 50 U/ml penicillin/streptomycin (PenStrep; Invitrogen), and maintained at 37°C in a 5% CO2 humidified atmosphere for 24 h.

4.1.2 Extraction and culturing of primary microglia

Isolation of CD11b+ cells was performed on the brain tissue of 3- to 5-day-old mice. Mice were euthanized through decollation.

The skull was opened, and the brain was carefully extracted. The cerebellum and olfactory bulb were removed and the remaining tissue was manually minced in HBSS using scalpels and then homogenized with pipets. Following this, the tissue was dissociated into a cell suspension using the MACS®Neural Tissue Dissociation Kit (P) (Miltenyi Biotech) following the manufacturer's protocol.

To sum up, the tissue was digested progressively using enzymes supplemented with the kit at 37°C and alternately homogenized with the gentleMACS® Dissociator (Miltenyi Biotech). Finally, the homogenate was filtered through a 70 μm mesh filter, centrifuged (1300 rpm, 10 min) and suspended in ice cold MACS buffer (PBS, pH 7.2, 0.5% FBS). Subsequently, the cells were incubated with 50 μl magnetic CD11b+ MicroBeads (Miltenyi Biotech) per gram and incubated for 15 min at 4°C. After a washing step, the cell suspension was suspended in 1500 μl of MACS buffer and, separated over LS magnetic columns (Miltenyi Biotech), placed in the MidiMACS® magnetic separation unit (Miltenyi Biotec). Cells were counted, 10^6 cells were plated per well in a 24-well plate containing DMEM supplemented with 10% FBS and 50 U/ml

PenStrep and maintained at 37 °C in a 5% CO2 humidified atmosphere for 24 hours prior to the following experiments.

4.1.3 *In vitro* IL-1β protein expression after inflammasome activation

To quantify the effects of the Ebselen treatment *in vitro* on NLRP3 inflammasome-dependent (IL-1β) protein expression, peritoneal macrophages from wt (wild type) mice were extracted as described and 10^6 cells were plated in a 24-well plate.

In order to activate the NLRP3 inflammasome *in vitro*, LPS, acting over TLRs, was used as a first signal and adenosine-tri-phosphate (ATP) as a second sterile inducer [146]. Then, 24 h after the extraction and plating of peritoneal macrophages, the culture medium was discarded and LPS (1 μg/ml) with 50 μM Ebselen or vehicle (DMSO) diluted in culture medium was added to stimulate the cells. An equivalent amount of PBS in medium was used as negative control for stimulation. After 5 h of incubation (37°C, 5% CO2), 5 mM ATP was added for 30 min with 50 μM Ebselen or vehicle (DMSO), then the supernatants were collected and measured for protein content with the ELISA required by the manufacturer's protocol (eBioscience).

To quantify the effects of Ebselen *in vitro* on primary microglia, the cells were planted as described above. The following day, the culture medium was discarded and LPS (0.1 μg/ml) with 50 μM Ebselen or vehicle (DMSO) diluted in culture medium was added to stimulate the cells. An equivalent amount of PBS in medium was used as negative control for stimulation. After 3 h of incubation (37°C, 5% CO2) 1 mM ATP was added for 3 h with 50 μM Ebselen or vehicle (DMSO), then cell supernatants were collected and measured for protein content with the ELISA required by the manufacturer's protocol (eBioscience) and as described for the peritoneal macrophages.

4.2 *In vivo* experiments

4.2.1 Animal handling

As a model for obesity and the metabolic syndrome we used 90-day-old male C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME), which were housed separately in a controlled environment (12-h daylight cycle, lights off at 6 p.m.) with free access to food and water. We

chose only males, because the metabolism of individual females differs considerably depending on their menstrual cycle. The mice were fed a control chow (D12450J) or high-fat chow diet (Rodent Diet with 60% kcal% fat, D12492, Research Diets) for different periods of time.

The experiments were carried out in accordance with national animal protection guidelines, after approval by the Landesamt für Gesundheit und Soziales in Berlin (LaGeSo Berlin).

4.2.2 Phenotypic characterization

For phenotyping, the mice were weighed every week. In addition, food consumption was measured, and new food was added to stop the high-fat food becoming greasy. In the experiment where Ebselen was applied through drinking water, the watering bottles had special anti-drip lids (Tk100 Watering tube, ball closure 100 mm, 3125052, Bioscape) to prevent spillage of the drug and to ensure correct dosage. The water consumption of the mice was, therefore, also measured. The weekly energy intake was calculated using the following equation:

$$Weekly\ energy\ intake = gram\ of\ weekly\ food\ intake * specific\ caloric\ density$$

The development of the bodyweight was compared to the initial bodyweight as a percentual bodyweight change:

$$Percentage\ bodyweight\ change = \frac{actual\ bodyweight}{initial\ bodyweight}$$

4.2.3 Intraperitoneal glucose tolerance test

The intraperitoneal glucose tolerance tests were performed monthly. Glucose resistance in the mice had to be tested on an empty stomach. Considering the different night/day rhythm of rodents, the food was withdrawn at about 7 a.m. In the experiment where Ebselen was administered through the drinking water, the water was changed to prevent the metabolism of the mice being influenced by the drug or its vehicle. After 6 hours of fasting, the basal glucose level was measured using the blood sugar meter ContourXT with the Contour Next sensor stripes from Bayer, with a maximum threshold of 600 mmol/ml of glucose. The mice were injected intraperitoneally with either 0.002g glucose per g bodyweight or with 0.001g glucose per g bodyweight. The blood sugar levels were taken from the tail veins at intervals of 0, 15, 30, 60, and 120 min after insulin injection. At each time point, the blood glucose was measured

twice. If the difference was higher than 10%, a third measurement was taken. The average was then calculated. In cases where the blood sugar value exceeded the maximum value measurable by the blood sugar meter, 600 mmol/dl was taken as the amount.

To evaluate the insulin resistance, the area under the curve was calculated using the following equation:

$$AUC_{0-t_n} \approx \frac{1}{2}\sum_{i=1}^{n-1}(t_{i+1} - t_i)(C_i + C_{i+1})$$

4.2.4 Measuring of body composition

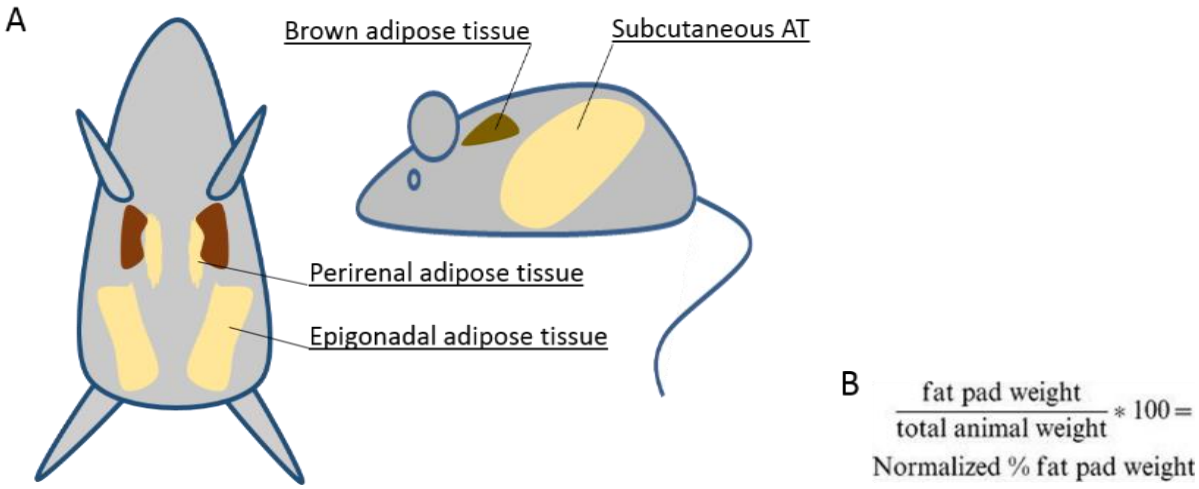

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Figure 2: *Different fat compartments of a mouse.* ***A*** *The white adipose tissue is divided into subcutaneous pads and visceral pads. The main white adipose tissue (WAT) pads are comprised of the epigonadal adipose tissue. Further perirenal adipose tissue was collected. Brown adipose tissue (BAT) is distributed throughout the adipose tissue with the main BAT depot in the interscapular region, which was dissected. As a subcutaneous fat pad, the posterior subcutaneous fat depot was collected.* ***B*** *equation for the calculation of normalized fat pad weight.*

Body composition, including lean mass, fat and free fluid, was measured by ^{1}H-magnetic resonance spectroscopy, using a body composition analyzer (minispec LF50, Buker), in the *in vivo* experiments in which the mice were treated with Ebselen via drinking water. The absolute and relative weight of the fat and lean mass were calculated.

After sacrifice, the different fat compartments from one side - gonadal fat, perirenal fat, subcutaneous fat and brown fat tissue - were dissected and weighed. For the brown fat, the fat compartments between the scapulas were taken. Since it is not possible to collect all subcutaneous fat, the fat pad of the back was taken, as marked in the picture. The amount of

adipose tissue from each depot was measured in absolute weight and was also normalized to the bodyweight of each animal using the equation shown (**Figure 2B**).

4.2.5 Measuring energy expenditure and locomotor activity with metabolic cages

Energy expenditure was measured twice during the experiments where Ebselen was administered through drinking water using a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany). The mice were given 48 h prior to the beginning of the experiment to get used to the drinking bottles. O_2 and CO_2 measurements were taken every 33 min, while the mice were given ad lib. access to food and water. VO_2 consumption and VCO_2 production in individual mice were measured using metabolic chambers, and the respiratory exchange ratio (RER) was calculated to reflect energy expenditure. Because of possible initial stress related to transfer to the chambers, only the last 24 h (of approximately 48 h in total) of measurements were used to calculate the respiratory quotient, determined as the ratio of VCO_2 to VO_2 ($RER = VCO_2/VO_2$). The energy expenditure was calculated from the RER and gas exchange data [$Energy\ expenditure = (3.815 + (1.232 * RER) * VO2$] and normalized to lean mass [46] as measured by the body composition analyzer. The total energy expenditure (TEE) is the sum of the means of hours of one day.

The physical activity was measured by an infrared beam system integrated into the LabMaster system. Total activity (beam breaks) in the *X*, *Y*, and *Z* axes was stored every 33 min. The system is designed to differentiate between fine motor movement (defined as a single *X*- or *Y*-axis beam break), ambulatory movement (defined as the simultaneous breaking of two adjacent *X* or *Y* beams), and rearing (defined as the breaking of the *Z*-axis infrared beam). For the total locomotor activity, all measurements of the last 24 h were added together. There were always 44 summands. The total locomotor activity was further divided into nighttime and daytime (nighttime: 6 p.m.- 6 a.m., 22 summands).

In the calculation of locomotion and energy expenditure, only animals that consumed more than 1 g of food and 1 ml of water were included.

4.2.6 Application of Ebselen *in vivo*

To test the effect of Ebselen *in vivo* in the model of high-fat diet-induced obesity, Ebselen was first dissolved in 0.5 mM dimethylsulfoxide (DMSO) and then slowly further diluted in 25%

w/v hydroxypropyl β-cyclodextrin (termed β-cyclodextrin), which served as a vehicle. The vehicle was permanently shaken at 50° Celsius.

120-day-old male mice were treated with 30 mg/kg of Ebselen or the vehicle by i.p. injection three times a week (3x10 mg/kg), or the same amount of Ebselen or vehicle diluted in drinking water. At the end of the experiments, the mice were killed using dry CO2. Initially, blood was withdrawn directly from the heart and then the mice were perfused with 20 ml ice cold PBS.

4.3 Immunohistochemical evaluations

Immunohistochemical staining was used to detect peritoneal macrophage cells (CD68) in order to evaluate the percentage of CD68 positive cells in the cell culture.

For staining, the cells were cultured on coverslips and incubated overnight. The coverslips were transferred to a sterile 6-well plate and incubated for 30 min with 4% PFA for the fixation of the cells. Following this, they were washed 3 times for 5 min with PBS and blocked with 10% normal goat serum (NGS) in PBS containing 0.3% Triton-X-100 (PBS-TX) for 1 h at RT on a shaker. Next, the blocking solution was discarded, the primary AB in 5% NGS in PBS-TX in a 1:500 dilution was added, and the cells were incubated overnight at 4 °C. The next day, the sections were washed three times with PBS and incubated with the secondary AB Cy3 in 5% NGS in PBS-TX for 60 min at RT on a shaker. After another washing round (3x PBS), sections were mounted on a glass cover slide, which was bathed twice in xylene for 1 min before covering, using Roti®-Histokitt II mounting medium.

Fluorescent sections were imaged using a confocal laser-scanning microscope (Leica). Three different plates, with 3 replicates, were used to quantify the purity of the peritoneal macrophage cell culture.

Primary AB	Host	Dilution	Company	Secondary AB	Host	Dilution	Company
CD68	Rat	1:500	Serotec	Cy3	Donkey	1:300	Jackson

Table 1:AB use for staining of peritoneal macrophages

4.4 Quantification of protein expression

4.4.1 Protein extraction from WAT and blood plasma

To determine IL-1β protein levels in the WAT of the mice where Ebselen was administered via drinking water, WAT from the gonadal fat compartment from mice transcardially perfused with PBS was extracted, weighed and snap frozen in liquid nitrogen. After weighing, the piece was homogenized in 1 µl/mg radioimmunoprecipitation assay buffer (RIPA; 50 mM Tris-HCl (pH 7.5), 150mM NaCl, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) containing protease inhibitor (Roche, 1 tablet per 50 ml). Homogenization was performed mechanically using syringes with cannulas of decreasing diameter (20, 23 and 27G) followed by an incubation time of 30 min on ice. After centrifugation (30 min, 12000 g, 4°C), supernatants, without the lipid phase, were snap frozen in liquid nitrogen.

The protein concentration of each sample was determined with the QuantiproTM BCA Protein Assay Kit (Pierce) using 1:10, 1:20 and 1:50 dilutions with the Photometer Tecan Infinite® 200M (Tecan) according to the manufacturer's protocol. This was followed by an analysis of the concentration of IL-1β using ELISA as required by the manufacturer's protocol (eBioscience). Using the same protocol, the IL-1β concentration of the blood plasma was analyzed using ELISA.

4.4.2 Quantification of IL-β

To determine IL-1β protein levels in the supernatant, serum or white adipose tissue, an enzymatic immunosorbent assay (ELISA) was performed following the manufacturer's protocol (eBioscience). As technical replicates for the cell culture experiments, 100 µl of supernatant was diluted to fit into the range of the standard curve. Absorption was measured at 450 nm and 570 nm (for wavelength correction) on a microplate reader (Infinite® 200M, Tecan) and analyzed using the MagellanTM Software.

4.5 Statistical analysis

Differences between groups were analyzed using GraphPad® Prism. All data was expressed as means ± standard deviation (SD). For comparisons of the means between two groups, statistical analysis was performed by applying the t-test in GraphPad Prism 9. Comparisons of the means

among more than two groups were made using a one-way or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons post-test in GraphPad Prism 9, as indicated in the figure legends. Results were displayed as mean values +/- standard deviation (SD).

P values of less than 0.05 were significant and were indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

The log-rank test was used to compare survival according to the different groups. P values of less than 0.0166 were significant and were indicated as follows: * $p < 0.0166$, ** $p < 0.0033$, *** $p < 0.00033$. The significance level was set at 0.0166, according to the Bonferroni corrected threshold, dividing the original p by 3, because of the 3 groups being compared with each other.

5 Results

5.1 Aim 1: Ebselen inhibits the inflammasome *in vitro*

As described in the introduction, the NLRP3 inflammasome plays a pivotal role in the aggravation of obesity itself and in the development of the metabolic syndrome. Ebselen, a selenium organic compound, has been identified as a specific inhibitor of the NLRP3 inflammasome (unpublished data, Veit Hornung from the University of Bonn).

Before applying Ebselen *in vivo* in our HFD obesity model, we wanted to ensure that it inhibits the main product of the NLRP3 inflammasome, IL-1β. Since the inflammasome is activated in macrophages in the adipose tissue, liver, and other organs in obesity [43] and also in microglia in the CNS, I tested the inhibitory potential of Ebselen on both cell types.

5.1.1 Ebselen inhibits IL-1β secretion in activated peritoneal macrophages

As described previously, before the products of the NLRP3 inflammasome - IL-1β and IL-18 - are secreted, the inflammasome must be activated. This requires 2 steps.

Cellular priming through nuclear factor NF-κB activation leads to the induction of proforms of IL-1β and IL-18 and the upregulation of NLRP3 itself, a step that is required for NLRP3 activation *in vitro* [37]. Since LPS is the most commonly used and researched priming signal in *in vitro* experiments in current literature, I used it as a reference in my *in vitro* experiments [30, 43, 49]. The inflammasome itself then regulates the proteolytic processing of pro-IL-1β. The activation step is in response to another pro-inflammatory stimuli. In the following experiments, ATP was used as an NLRP3 inflammasome activator. In various publications, the NLRP3 inflammasome was stimulated in peritoneal macrophages with LPS followed by ATP. While various time points have been used in different publications, we chose a well-established time course, as described previously [34].

We first tested whether Ebselen inhibits the NLRP3 inflammasome in general. In order to do this, the peritoneal macrophages were incubated with LPS as the priming stimulus, followed by ATP stimulation to activate the inflammasome (**Figure 3A**). The supernatants were analyzed with an ELISA for IL-1β.

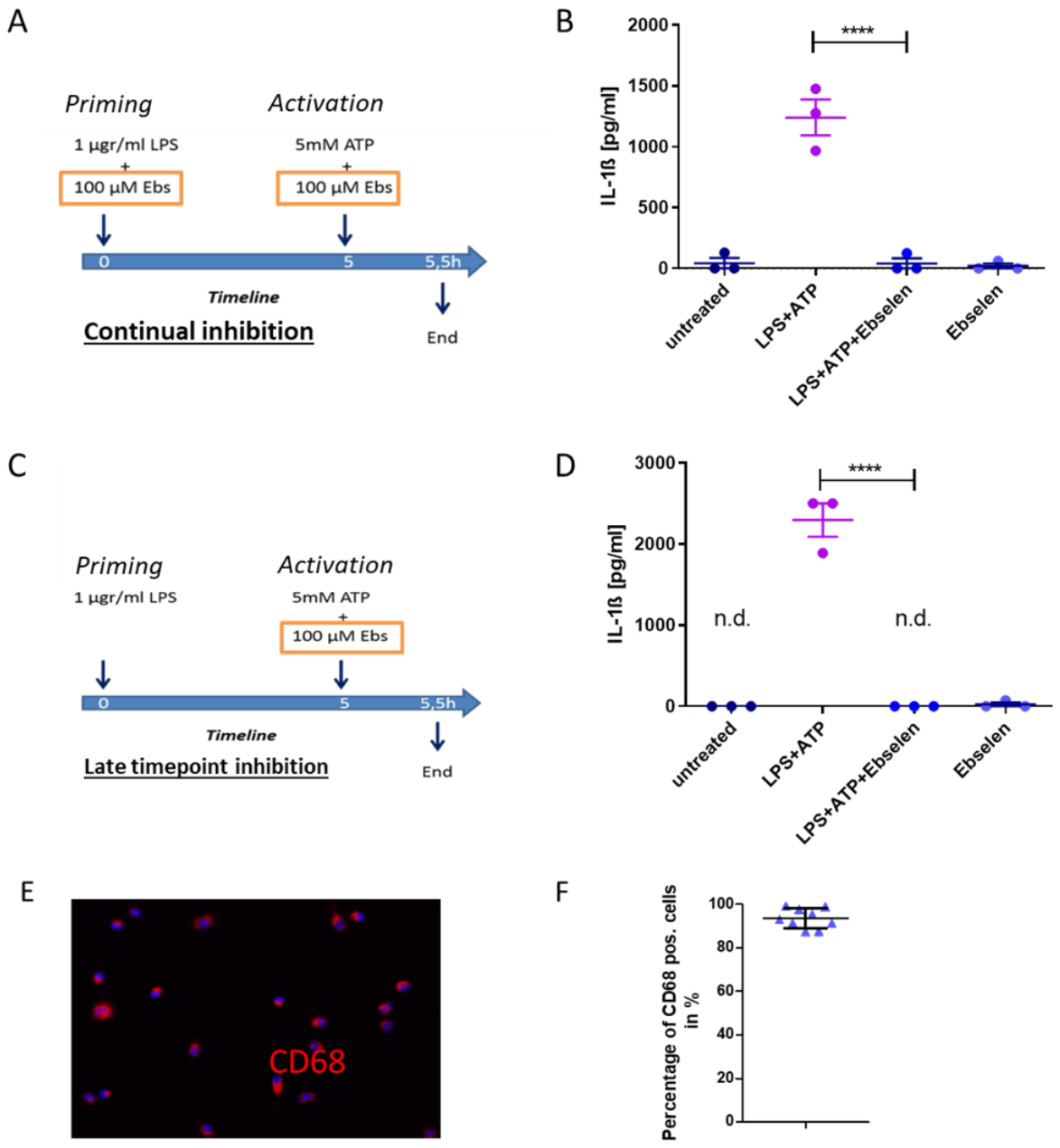


Figure 3: *Ebselen reduces IL-β secretion in cultured peritoneal macrophages.* ***A*** *Experimental setup of the "whole time" inhibition. Untreated primary peritoneal macrophages were left untreated for 5.5 h (medium change after 5 h). In the second group the cells were treated with LPS for 5 h and subsequently treated for 30 min with ATP to activate the inflammasome. In the third group, Ebselen was added during the priming step (LPS) and during the activation step (ATP). The last group was only treated with Ebselen.* ***B*** *Stimulated peritoneal macrophages secrete IL-1β in the supernatant. The production of IL-1β is potently inhibited by 100 µM Ebselen applied during the priming and the activation step. (N = 3 with three technical replicates).* ***C*** *Experimental setup of the "late time point" inhibition: Primary peritoneal macrophages were left untreated for 5.5 h (medium change after 5 h). In the second group the cells were treated with LPS for 5 h and then treated for 30 min with ATP. In the third group, Ebselen was added only during the activation step (ATP). The last group was only treated with Ebselen after 5 h for 30 min, and neither LPS nor ATP were added. (N = 3 with three technical replicates).* ***D*** *Stimulated peritoneal macrophages secrete IL-1β in the supernatant. The production of IL-1β is potently inhibited by 100 µM Ebselen, applied during the activation step. (N = 3 with three technical replicates).* ***E*** *Representative picture of plated cells from peritoneal macrophage cell culture stained with CD68-Cy3.* ***F*** *Stereological cell count of CD68+ stained cells shows that 93.59% ± 4.5 of cells are positive for CD68 (n=9). Each dot represents the mean of three replicates, mean and SD shown. Statistics: one-way ANOVA with Bonferroni's Multiple Comparison Test).*

Little IL-1β was detected in untreated peritoneal macrophages (43.22 pg/ml± 74.86), which indicates that they were in a resting state. Following LPS and ATP stimulation to activate the NLRP3 inflammasome, a significant increase of IL-1β was measured in the supernatant (1239 pg/ml ±255.2; P=****). This increase was reversed by the application of Ebselen during LPS application and during the administration of ATP (41.19pg/ml ±71.35, P=****), resulting in the same basal level as the unstimulated cells (**Figure 3B)**.

To prove that Ebselen does not have any effect on unstimulated cells, it was also added to unstimulated peritoneal macrophage cells. In this case, Ebselen did not change the IL-1β secretion (mean 20.10pg/ml ±34.81).

To determine whether Ebselen specifically inhibits the activation step of inflammasome activation, in which the inflammasome complex is formed and caspase 1 is activated, leading to the cleavage and release of IL-1β, we conducted an experiment in which the time course was changed and Ebselen was exclusively added to the application of ATP, the activation step (**Figure 3C**). In this experimental setup, called "late time point inhibition", the same groups were selected as in the prior experiment, the only difference being the administering of Ebselen only after the priming with LPS.

The untreated cells produced an undetectable amount of IL-1β. With LPS and ATP stimulation, IL-1β secretion was detected (2296 pg/ml ±353.0). When adding Ebselen simultaneously with ATP, this effect was reversed. The untreated cells incubated with Ebselen at the late time point showed a slight but insignificant production and the release of IL-1β into the supernatant (24.53pg/ml ±42.49) (**Figure 3D**).

To ensure the purity of the peritoneal cell cultures, the cells were immunohistochemically labeled with CD68, an intracellular macrophage marker frequently used to identify murine macrophages [50] (**Figure 3E, F)**. DAPI was used as a counterstain. The number of CD68 positive cells was divided by the total number of cells. On average 93.59% ±4.5 of cells were CD68 positive cells.

5.1.2 Ebselen inhibits IL-1β secretion in activated microglia

New results have proved that a meta-inflammation in the brain, especially in the hypothalamus, occurs in association with obesity. Inflammasome activation plays a major role in the development of many different diseases, for example Alzheimer's.

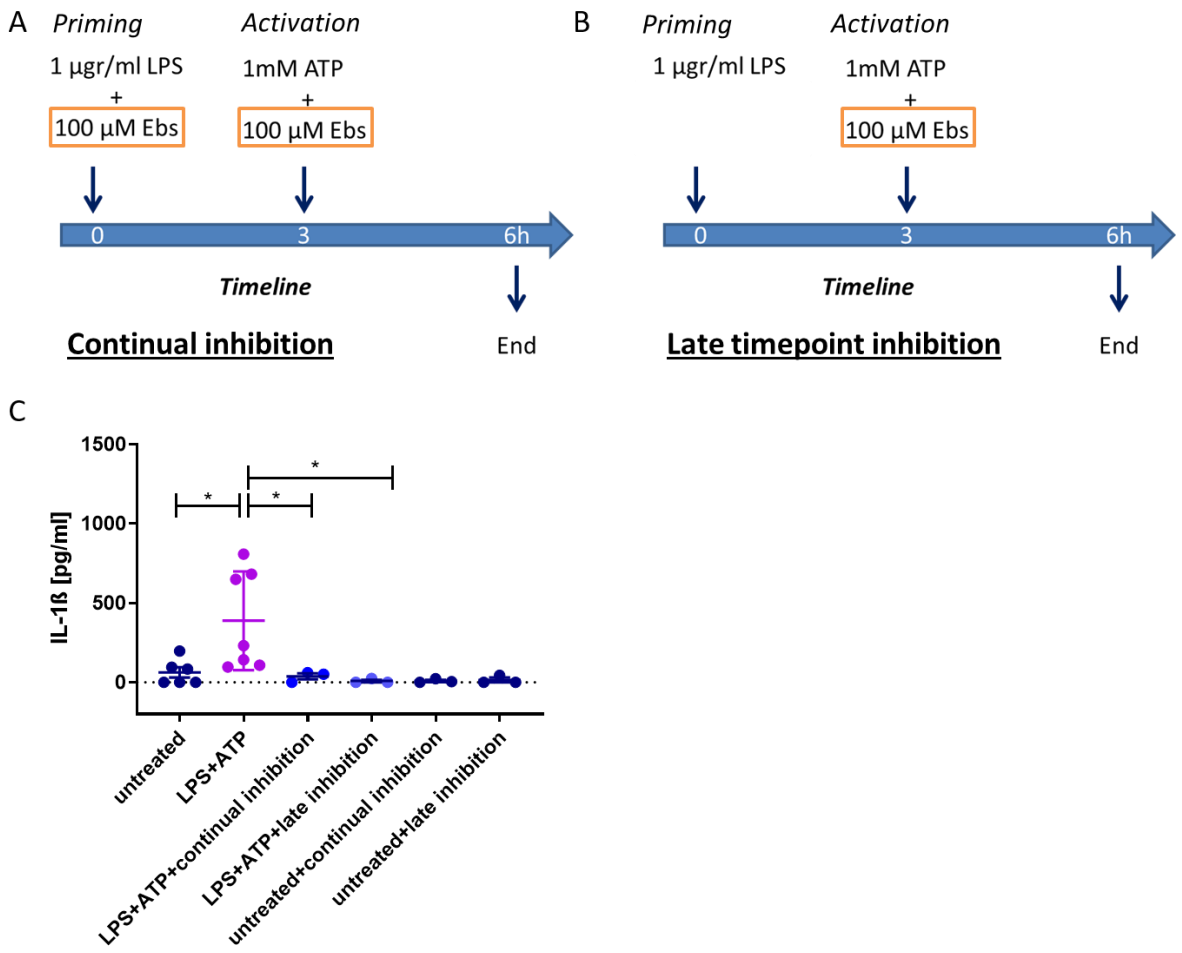


Figure 4: *Ebselen reduces the secretion of IL-β in cultured primary microglia.* ***A*** *Experimental setup of the "whole time" inhibition: Untreated cells: Microglia were left untreated for 6 h (medium change after 3 h), LPS+ATP: treated with LPS for 3 h and then treated for 3 h with ATP to activate the inflammasome. In the third group, Ebselen was added during the priming step (LPS) and during the activation step (ATP).* ***B*** *Ebselen was added only during the activation step (ATP) after 3 h in the LPS+ATP+late inhibition to the microglia. The fifth group was only treated with Ebselen for 6 h with medium change after 3 h in the late inhibition. The last group, untreated + late inhibition, was only treated with Ebselen after 6 h, and neither LPS nor ATP were added.* ***C*** *The production of IL-1β is potently inhibited by the administration of 100 µM Ebselen, applied during the priming and the activation step, as well as just being applied during ATP administration (LPS+ATP+late inhibition). Statistics: one-way ANOVA with Bonferroni's Multiple Comparison Test.*

Potential NLRP3 inflammasome inhibition, not only in peritoneal macrophages but also in microglia cells could, therefore, be of therapeutic importance. Whereas in microglia, LPS priming of IL-1β expression requires caspase-8 activation, this is not the case in macrophages [51]. It was, therefore, important to prove the efficacy of Ebselen in microglia as well.

Since different publications use different time courses for LPS and ATP stimulation for NLRP3 inflammasome activation in primary microglia cell culture, a time course had to be established [52, 53].

MACS-sorted microglia from Bl6 pups were used in the cell culture experiments. Multiple time courses were tested and IL-1β was measured in the supernatants using an ELISA. Every time

course showed detectable IL-1β release. The greatest Il-1β secretion was measured using the following time course: 3 h incubation of the cells with 0.1mg LPS in 1 ml medium followed by 3-hour stimulation of the cells with 1mM ATP. This was selected for subsequent experiments**.**

In the continual inhibition, Ebselen was administered at both time points (**Figure 4A**), while in the late time point inhibition, the Ebselen was only administered simultaneously with ATP at the late time point after 3 hours (**Figure 4B**). Little IL-1β was detected in untreated primary microglia cells (mean 62.77 pg/ml± 79.27), indicating that they were in a resting state (**Figure 4C)**. Following LPS and ATP stimulation, a significant increase of IL-1β was measured in the supernatant (388.0pg/ml ±310.5; p=*). This increase was inhibited through the application of Ebselen during the priming and activation step (37.53pg/ml ±32.97, P=*). The inhibition of IL-1β release was also achieved by adding Ebselen to the cells after 3 h (8.33pg/ml±14.43, P=*).

Ebselen did not change the IL-1β secretion in untreated cells during the continual inhibition (9.533pg/ml ±12.21) or during the late time point inhibition (14.83pg/ml ±25.69).

5.2 Aim 2: Ebselen in the context of HFD-induced obesity

After showing that Ebselen could inhibit IL-1β release in both peritoneal macrophages and microglia *in vitro*, the effect of Ebselen in the high-fat diet-induced obesity (HFD-IO) model was examined.

5.2.1 HFD-fed mice show a metabolic phenotype after 2 months

To be able to ameliorate the metabolic phenotype in our obesity mouse model, we first identified the time point of the establishment of a metabolic phenotype in our mouse model. The 6 black mice were fed either an HFD or a standard diet (CHOW), as described. After 30 days, an ipGTT was performed. After only 10 days, the HFD mice had gained more weight than the CHOW-fed mice (HFD: 115.4% ±9.748, CHOW: 99.6%±5.93, P=**). After 59 days, the HFD mice had an obese phenotype and weighed 38% more than the control group (114.5%±7.962 of initial body weight, HFD vs CHOW: P=***). (**Figure 5A**)

As described in other studies [56], the intraperitoneal glucose tolerance test was carried out by injecting 0.002g glucose per g of bodyweight. After 30 days of an HFD, the basal fasting

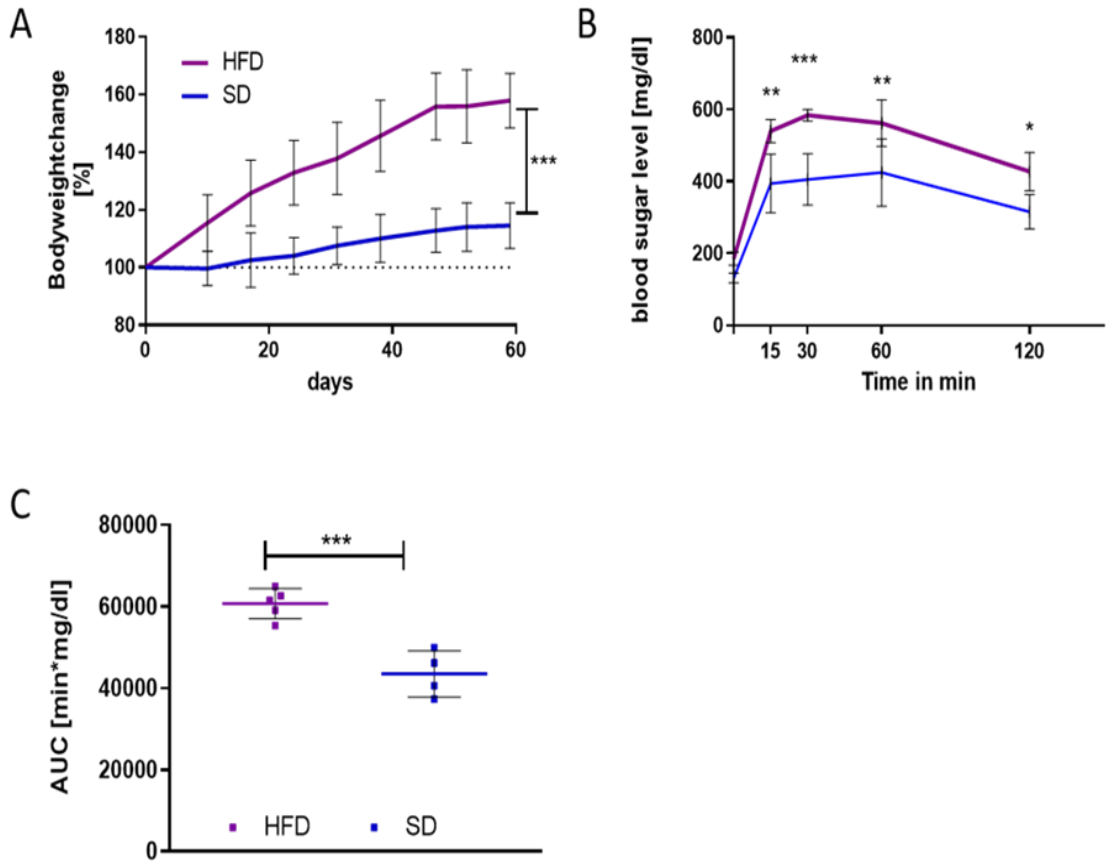


Figure 5: *HFD induced weight gain and impaired glucose tolerance only after 30 days.* ***A****: Comparison of bodyweight gain of mice fed with CHOW and HFD N=9-10, two-way ANOVA.* ***B, C:*** *Blood sugar levels and area under the curve in the ipGTT. High-fat feeding was associated with impaired glucose tolerance compared to normally fed mice after 30 days; p=***, n=4-5, Each dot represents one measured animal with the mean of 2-3 measured blood glucose levels at each time point. Statistics: two-way ANOVA with Bonferroni post-test, or t-test.*

glucose level was identical (HFD: 185.9 mg/dl±18.9, CHOW: 131.75 mg/dl±13.1). At 30, 60, and 120 min time points of the intraperitoneal glucose tolerance test, the HFD group had higher glucose levels (N=5, two-way ANOVA), **Figure 5, B**). The area under the curve was calculated. The mice developed glucose resistance only after 30 days on the high-fat diet. The HFD control group (60755 min*(mg/dl) ±1641. N=5) showed a significant increase of 39.5 % (t-Test; p=***) in the area below the curve compared with the standard diet control group (43553 min*(mg/dl) ±2815, N=4) (**Figure 5C**).

Since the Contour Next can only measure a maximum glucose value of 600 mmol/ml and this level was reached for 6 of the 9 mice in the ipGTT, a dosage of 0.01mg/gr was used with a 10% glucose solution for the ensuing experiments. Another study [49] showed that inflammasome activation in white adipose tissue requires 8 weeks of HFD feeding, so we therefore decided to run the experiments over at least 8 weeks. Since the HFD mice showed an obese phenotype and

glucose resistance after 1 month, we started with the main experiments, applying Ebselen to our HFD-IO mouse model.

5.2.2 Intraperitoneal injection of Ebselen ameliorates glucose tolerance, but leads to a higher mortality in mice

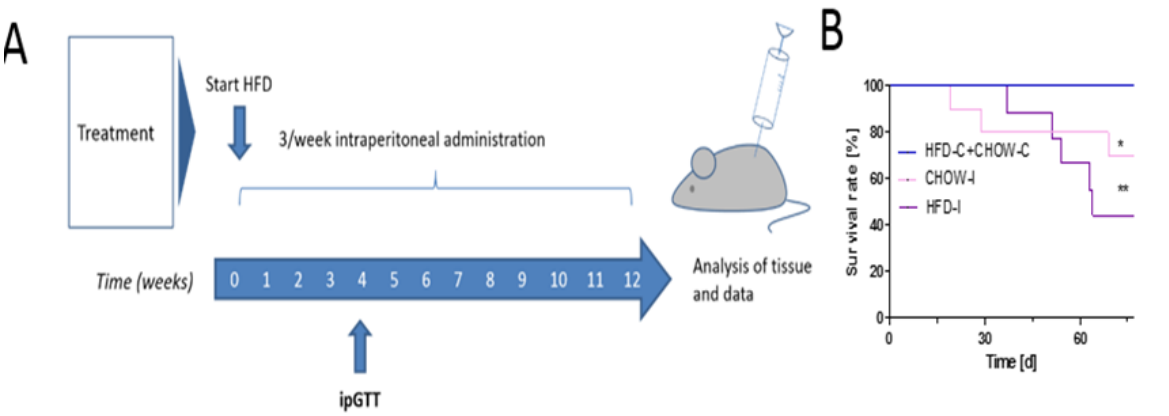
Groups	Diet	Treatment	Abbreviation
1	CHOW	Inflammasome Inhibitor	CHOW-I
2	HFD	Inflammasome Inhibitor	HFD-I
3	CHOW	Vehicle Control	CHOW-C
4	HFD	Vehicle Control	HFD-C

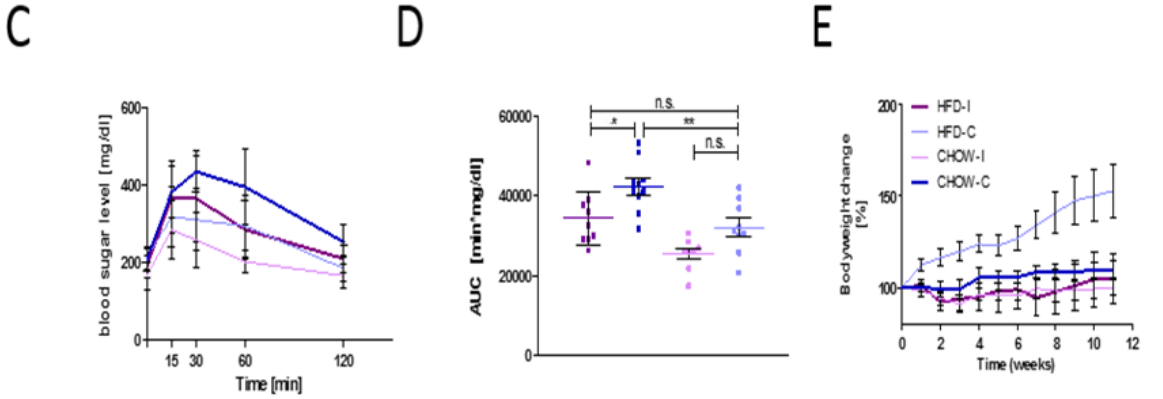
Table 2: *Experimental groups to test the effect of Ebselen in the HFD-IO model. Experimental setup: 90-day-old BL6 mice were fed an HFD or CHOW as control. Both the HFD and the CHOW groups were injected intraperitoneally 3 times a week, either with the inflammasome inhibitor Ebselen or with the vehicle*

For the *in vivo* inhibition of the NLRP3 inflammasome, Ebselen was dissolved at 0.5 mM in DMSO and further in 25% β-cyclodextrin, which served as a vehicle for the Ebselen, which has a very low water solubility [57]. Ebselen was intraperitoneally injected 3 times per week at 10 mg/kg. This amount of Ebselen has also been used in metabolic rat models, where it had positive effects in the periphery [58, 59]. In addition, this concentration of Ebselen has been shown to influence the CNS when administered peripherally [60]. For the control, the same amount of DMSO was dissolved in the vehicle and injected intraperitoneally. The DMSO concentration was below 0.5%, a concentration which is non-toxic [61]. Likewise, the vehicle concentration had been proved to be tolerable in mice over long treatment periods and non-toxic in mice and rabbits [62, 63].

As lean control groups, groups were fed a standard diet and treated either with the vehicle or the inflammasome inhibitor (**Table 2**). During the experiment, an ipGTT was performed after 4 weeks and bodyweight gain and food intake were measured weekly (**Figure 6A**).

Surprisingly, 55.5% of the HFD-fed mice treated with Ebselen and 30% of the CHOW-fed mice treated with Ebselen had died by day 86, whereas all animals injected with the vehicle stayed healthy (**Figure 6B**). The survival rate of the mice injected with Ebselen differed significantly from that of the mice injected with the vehicle (HFD-I vs. HFD/CHOW-C: p=***; CHOW-I vs. HFD/CHOW-C: p=*, log-rank test, Bonferroni post-test K=3). There was no significant difference in the survival rate of the two groups injected with Ebselen, suggesting that the cause


A
Treatment
Start HFD
3/week intraperitoneal administration
Time (weeks)
0 1 2 3 4 5 6 7 8 9 10 11 12
ipGTT
Analysis of tissue and data
B
Survival rate [%]
HFD-C+CHOW-C
CHOW-I
HFD-I
Time [d]


C
blood sugar level [mg/dl]
Time [min]
D
AUC [min*mg/dl]
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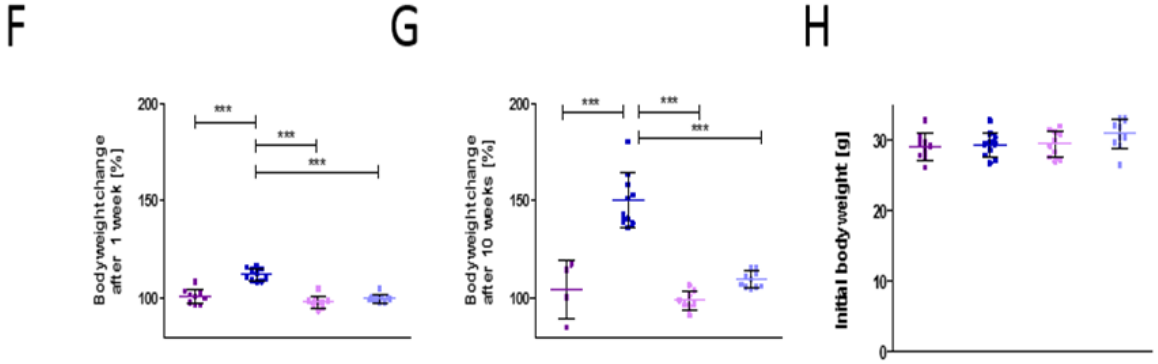
E
Bodyweightchange [%]
HFD-I
HFD-C
CHOW-I
CHOW-C
Time (weeks)


F
Bodyweightchange after 1 week [%]
G
Bodyweightchange after 10 weeks [%]
H
Initial bodyweight [g]

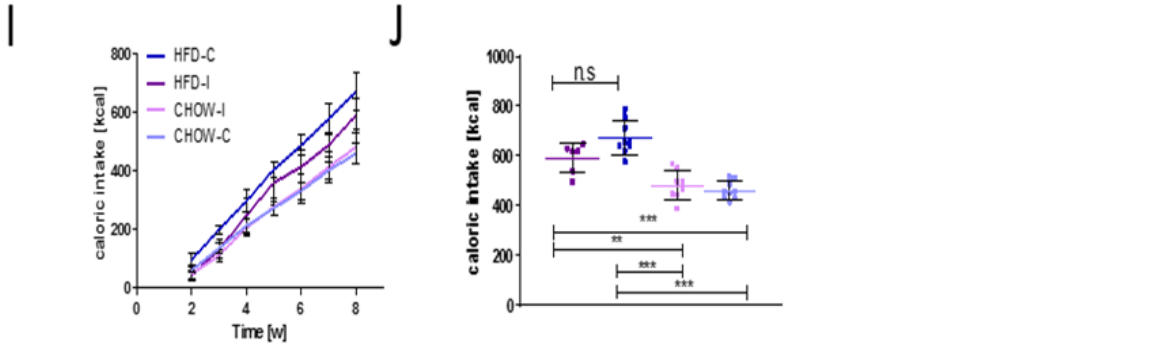

I
caloric intake [kcal]
HFD-C
HFD-I
CHOW-I
CHOW-C
Time [w]
J
caloric intake [kcal]
n.s

Figure 6: *Administration of Ebselen through intraperitoneal injections leads to a higher mortality in mice, but also to an improved glucose tolerance and less weight gain in HFD mice.* ***A*** *Experimental setup: Different groups were treated three times per week with Ebselen or vehicle and fed HFD or CHOW. After one month, ipGTT was performed. Weight and food intake were measured weekly. Serum was collected at the endpoint of the analyses and WAT was collected.* ***B*** *Kaplan-Meier curve showing mortality of mice administered Ebselen by intraperitoneal injections. Injection of Ebselen leads to higher mortality in the HFD and CHOW groups compared with the Control groups (N=9-10; log-rank test).* ***C,D*** *Blood sugar levels during the ipGTT after 4 weeks. High-fat feeding was associated with impaired glucose tolerance compared to chow-fed mice, Ebselen-treated HFD fed mice did not develop glucose resistance; N=8-10.* ***E,F,G*** *Comparison of weight gain.* ***F*** *Comparison of weight gain after one week. HFD Control mice gain more weight than the other groups; N=9-10.* ***G*** *Comparison of weight gain after 10 weeks. HFD Control mice weigh more than the other groups. Ebselen prevents weight gain when fed HFD; N=4-10.* ***H*** *Initially the bodyweight of the mice did not differ; (N=9-10).* ***I*** *Cumulative caloric intake of the different groups.* ***J*** *Cumulative caloric intake of week 2 to week 8. The Ebselen-treated mice do not eat significantly less than the HFD Control group. Each dot represents one measured animal or the mean of 2-3 measured blood glucose levels of an animal at each time point. Statistics: log-rank test, two-way ANOVA or one-way ANOVA with Bonferroni post-test.*

of death was due to the i.p. Ebselen injections rather than the diet. Postmortem autopsies revealed a swollen and hardened peritoneum, as well as signs of peritonitis. The different organs were attached to each other, so that their normal movement was not possible. There was also a solid white powder, which we assumed to be the precipitated Ebselen. Vehicle-injected mice were healthy and showed no adverse side effects. The postmortem biopsy did not show any pathological signs in the peritoneum.

Despite the higher mortality rate in the groups injected with Ebselen, the ipGTT was performed after 4 weeks of treatment (**Figure 6C**). After 4 weeks, the HFD led to an insulin resistance. The HFD-C group (42450 min*(mg/dl) ±6401) showed a significant 31.9% increase (p=**) in the AUC compared with the CHOW-C group (32181 min*(mg/dl) ±6688) (**Figure 6D)**. In contrast, the HFD group injected with Ebselen (AUC=34352 min*(mg/dl) ±6866) did not show a significant increase compared to the CHOW-C group. The injections of Ebselen improved the glucose tolerance in the HFD mice, reducing the AUC significantly (p=*) by nearly 20% compared with the HFD-C group. In lean mice, the injections of Ebselen did not alter the glucose uptake (CHOW-I: AUC=25467 min*(mg/dl) ±4118, CHOW-I vs. CHOW-C: p=ns). In mice injected with Ebselen, four weeks of HFD led to an impaired glucose tolerance (CHOW-I vs HFD-I: p=*) Only after four weeks of treatment with Ebselen via intraperitoneal injections was the metabolic phenotype reversed, which impeded the development of glucose resistance. Since many animals had died, we decided not to perform a second ipGTT after 2 months of treatment.

In addition to the ipGTT, bodyweight was measured weekly, since the mice with the genetic NLRP3 knockout lost bodyweight in DIO and a pharmacological inhibition could lead to the same changes. Furthermore, a reduction of bodyweight could explain the improved glucose sensitivity of the HFD-I group. As anticipated, the HFD-C group developed an obese phenotype

(**Figure 6E**). Since mice died during the experiments, a two-way ANOVA could not be applied to the data. After just 1 week of treatment (**Figure 6F**), the HFD-C were obese (HFD-C: 112.2%± 3.186. CHOW-C: 100.1%± 2.104, p=***). The intraperitoneal injections of Ebselen obviated weight gain in mice fed with an HFD (HFD-I vs. CHOW-C: ns; HFD-I: 101%±3.747, vs HFD-C p=***). Ebselen injection reduced the bodyweight by 10% when fed an HFD. The injection of Ebselen did not change the bodyweight in the CHOW groups (CHOW-I: 98.16%±2.993; CHOW-I vs. CHOW-C: p=ns). After 10 weeks (**Figure 6G**), the differences in bodyweight increased. The HFD-C group (150.3%±13.96) weighed 37.3% more than the CHOW-C group (109.5%± 4.316) (HFD-C vs. CHOW-C: P=***). The injected mice fed an HFD (HFD-I: 104.5%±14.96) weighed 30.5% less than the HFD-C group (P=***). The injection of Ebselen did not change the bodyweight in CHOW-fed, lean subjects (CHOW-I=99.11±4.79). Since the percentual bodyweight change was calculated, the initial BW (bodyweight) of all mice, which was identical (**Figure 6H)**, was compared.

Since the injection of Ebselen prevented the mice from becoming obese, the reason for this had to be examined. In order to distinguish whether the change in bodyweight was due to less caloric intake or to enhanced energy expenditure, the food intake of the mice was measured weekly. The caloric intake of all groups was calculated by the amount of food they ate multiplied by 5.24 kcal/gr for the HFD or 3.85 kcal/gr for the CHOW. Unfortunately, no data for the food intake could be acquired during the first week, therefore the cumulative food intake starts at week 2 and ends after 8 weeks (**Figure 6I**). After 8 weeks (**Figure 6J**), the HFD groups had ingested more calories than the groups fed a CHOW diet (HFD-I 591.493kcal±59.529; HFD-C 672.653kcal±67.076, CHOW-I 483.127kcal±58.827; CHOW-C 462 kcal±35.351). HFD-mice treated with Ebselen did not eat less than the control HFD group (p=0.0623). The food intake in mice fed with a standard diet was not changed by injecting ebselen (p=0.99)

When we examined the mice, we decided not to analyze the tissue further, since under these circumstances we could not ensure that the weight loss and improved glucose tolerance was not due to the local inflammation of the bowel. To test therapeutic usage of Ebselen in the context of DIO further, the treatment paradigm would have to be changed, since i.p. injections of Ebselen caused local peritonitis and lowered the survival rate.

5.2.3 Ebselen in the context of HFD-IO, administered through drinking water

5.2.3.1 Ebselen can be administered orally in mice

Following alternative published protocols, we decided to apply Ebselen orally through drinking water to test the therapeutic possibility of NLRP3 inhibition in the context of obesity. Previous work had shown that the intake of Ebselen through the gut by oral gavage over a long period did not cause any side effects [64-66]. In a human study, Ebselen was consumed dissolved in drinking water. [67]

Since oral gavage could injure the mice and be more stressful for them, which could lead to a decrease in bodyweight, we dissolved Ebselen in drinking water. In order to use this method, prior to starting the experiment, it had to be shown that Ebselen was stable and effective after being dissolved in the vehicle and kept at room temperature for 1 week.

Therefore, an *in vitro* experiment with peritoneal macrophages was performed, in which the efficacy of freshly dissolved "new" Ebselen was compared to "old" Ebselen, which had been dissolved in a medium and stored for one week. The cells were cultured as previously described (see 4.1.1). First, Ebselen was dissolved in 0.5 mM DMSO and further in 25% β-cyclodextrin, which served as the vehicle. The vehicle was further diluted in RPMI medium to the final concentration of 100 μM, as in prior experiments. The "old" Ebselen was stored at room temperature with exposure to light for 1 week before being used in the cell culture.

In the *in vitro* experiment, the peritoneal macrophages were stimulated with 1 μg/ml LPS for 6 h followed by a 30 min ATP administration. Either the "old" Ebselen or the "new" Ebselen was applied. Ebselen did not lose its potency after being dissolved and stored at room temperature for one week **(Figure 7A).** Both the "new" (80.4pg/ml) and the "old" (105.9pg/ml) Ebselen decreased the amount of IL-1β secretion compared with the stimulated (623.2pg/ml). Since one-week-old Ebselen had the same effect as freshly dissolved Ebselen, we decided to administer Ebselen dissolved in drinking water to the mice.

As in the case of the previous *in vivo* experiment, the oral Ebselen treatment was started using 90-day-old male mice (**Figure 7C**). As described, Ebselen was dissolved at 0.5 mM in DMSO and then carefully further dissolved in 25% β-cyclodextrin, which served as the vehicle. The same weekly dose of Ebselen or vehicle was dissolved in drinking water as for the i.p. injections. The water was changed every week and special drip-proof water dispensers were

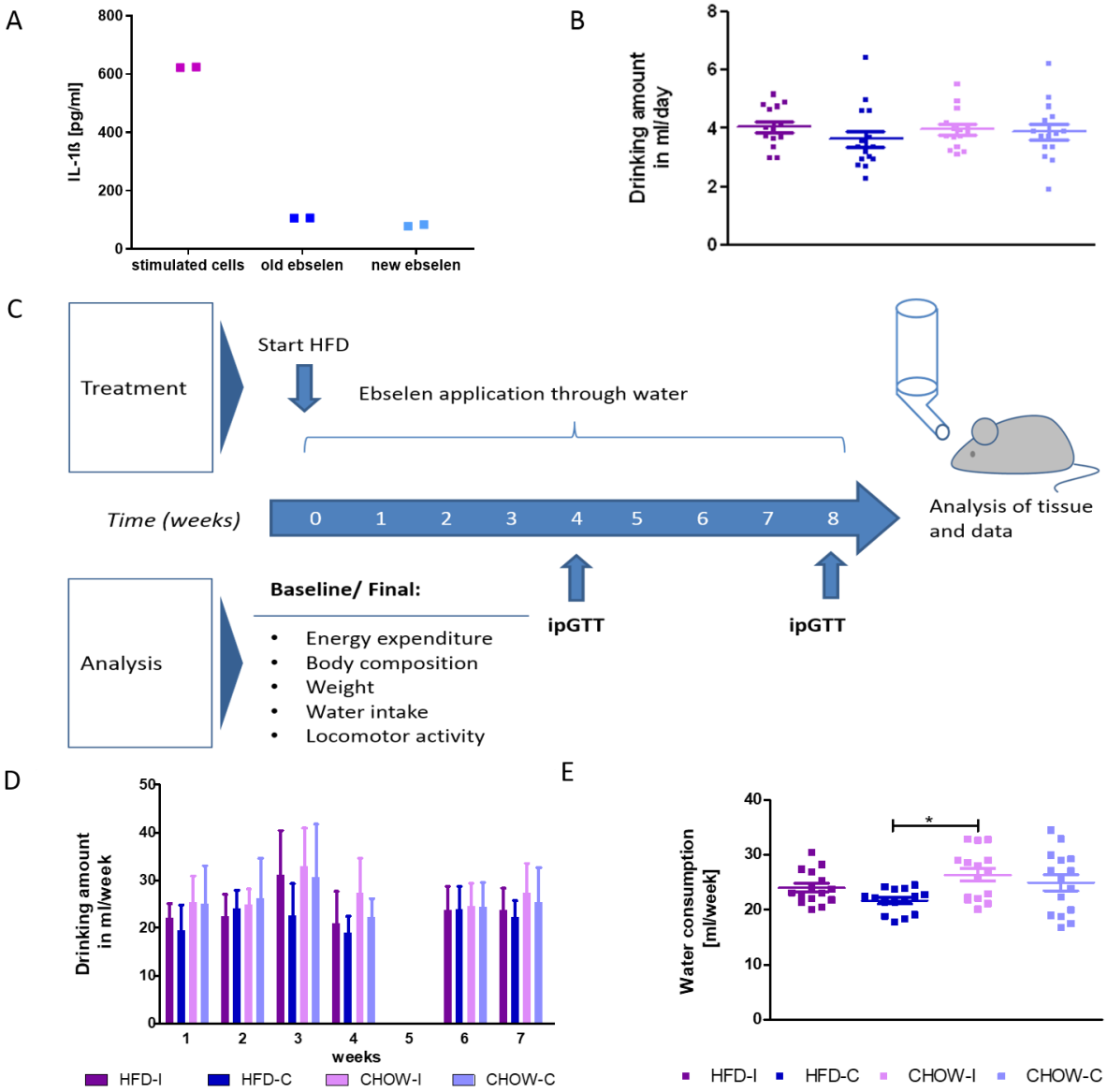


Figure 7: *Ebselen can be administered orally through drinking water.* ***A*** *Ebselen inhibits IL-1β after 1 week diluted at room temperature (old Ebselen) comparably to freshly diluted Ebselen (new Ebselen).* ***B*** *Initially, measured in the metabolic cages, the mice had the same fluid consumption (3.86 ml/day).* ***C*** *Experimental setup: Different groups were treated with Ebselen or vehicle dissolved in drinking water and fed HFD or CHOW. After 1 and 2 months, an ipGTT was performed. Weight, water and food intake were measured weekly. Serum was collected at the endpoint of the analyses, WAT was collected initially, and at the end point, water intake, locomotor activity and energy expenditure were measured in the metabolic cages.* ***D*** *Drinking amount was measured over the time, except for week 5.* ***E*** *The mean fluid consumption per week of the HFD-C group decreased significantly compared to the CHOW-I group, Ebselen treatment did not change the fluid consumption (P<0.001). Each dot represents one measured animal at each time point (N=15), with mean and SD. Because of water dripping, some data points are missing in some weeks. Statistics: one-way ANOVA with Bonferroni post-test.*

used, to minimize loss of water and to measure the exact drinking volume of each animal. Since the animals in this study were able to regulate their consumption of Ebselen via their water intake, fluid consumption was monitored over the time of the experiment. The necessary concentration of Ebselen in the drinking water was calculated by the daily water intake

measured in the metabolic cages before the start of the experiment and then adjusted according to the weekly water intake of the mice **(Figure 7B, D)**.

No difference between the water consumption of the groups was recorded in the measurements in the metabolic cages (**Figure 7B**). In the metabolic cages, the drinking bottles had special anti-drip mouthpieces so that the loss of water measured was identical to the amount of water drunk. The average water intake was 3.875ml/d ±0.8769. The different groups drank similar amounts of water (HFD-I 4.049ml/d ±0.7050; CHOW-I 3.631ml/d ±1.085 HFD-C 3.954ml/d±0.6645, CHOW-C 3.881ml/d ±1.003). All mice were CHOW-fed at the time of the initial measurements in the metabolic cages.

In week 5 of the experiment, the water consumption could not be measured due to the ipGTT, where the water with Ebselen or vehicle was substituted with normal water. For the same reason, the water measurements stopped after 7 weeks, since in the final metabolic cage measurement, the drinking bottles had to be exchanged 2 days prior to the experiment (**Figure 7D**). The HFD-C group drank less than the CHOW-I group during the experiment (**Figure 7E**). (HFD-I: 24.03ml/week ± 3.03; HFD-C: 21.65 ml/week ± 2.2; CHOW-I: 26.4 ml/week ± 4.48; CHOW-C: 24.92ml/week ± 5.7; HFD-C vs CHOW-I: P=*) The administration of Ebselen did not change the water consumption (HFD-I vs HFD-C: P=ns; CHOW-I vs. CHOW-C: P=ns).

All mice remained healthy throughout the experiment, with no signs of side effects. In contrast to the intraperitoneal injections, the oral application of Ebselen did not cause any deaths and a dosage of 30mg/kg/week was well tolerated by the mice over the period of 8 weeks.

In conclusion, a good alternative to the intraperitoneal injections was found, with *in vitro* tested stability and no signs of side effects.

5.2.3.2 Ebselen does not avert obesity when administered orally

After showing the stability of dissolved Ebselen exposed to light at room temperature for at least 1 week and adjusting the concentration of Ebselen to the drinking volume, the physiological responses of the mice after exposure to an HFD and Ebselen were examined through initial and final measurements in the metabolic cages, monthly ipGTT, and weekly bodyweight and food consumption measurements.

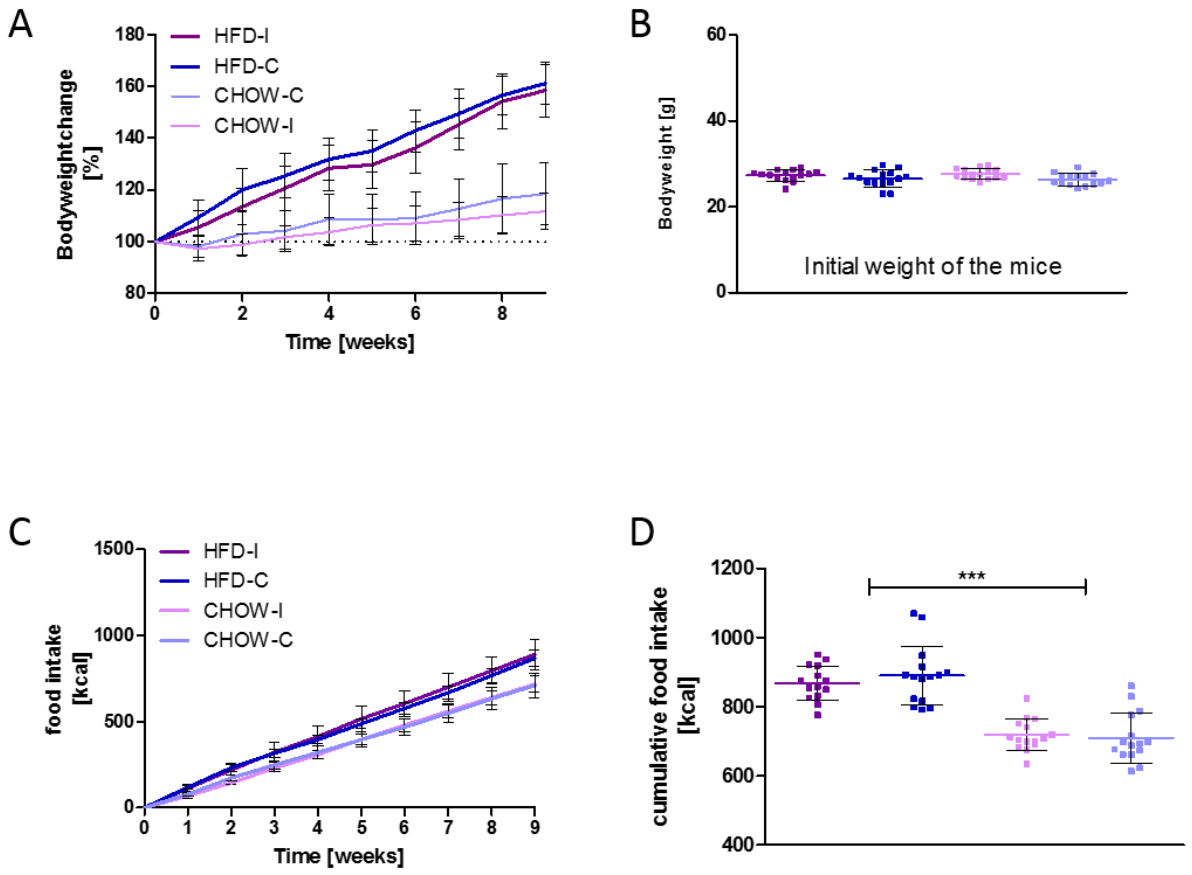


Figure 8: *Oral Ebselen treatment does not influence bodyweight change and caloric intake of mice.* ***A*** *Bodyweight change over the time course: Bodyweight change increases in all groups, HFD feeding leads to an obese phenotype after only 2 weeks, with no effects from the Ebselen treatment.* ***B*** *Initially, all groups had the same bodyweight.* ***C*** *Development of the cumulative caloric intake of the different groups over the time course, HFD feeding leads to higher caloric intake.* ***D*** *Average caloric intake per week during the experiment. HFD-fed mice consume more calories, Ebselen treatment does not change caloric intake. Each dot represents one measured animal at each time point (N=15), with mean and SD. Statistics: two-way ANOVA or one-way ANOVA with Bonferroni post-test.*

In our previous experiment, where Ebselen was administered intraperitoneally, the HFD-fed group treated with Ebselen gained significantly less weight than the HFD-C group. Stienstra [44] has shown that NLRP3 knockout mice and ASC knockout mice gain less weight when fed a high-fat diet. Hence, the weight difference between the HFD-C and HFD-I group could be due to the assumed pharmacological effect, the inhibition of the NLRP3 inflammasome, or due to an inflammation, such as peritonitis. Using the new method of Ebselen application, with which there were no visible signs of side effects, the weight homeostasis in the context of HFD was assessed.

During the experiments, the weight of the mice was measured weekly and the percentual body weight change was calculated as explained previously. A static weight gain could be seen in all groups (**Figure 8A**). In the chow-fed groups, this increase is common and due to age-related

bodyweight gain. After only two weeks, the change in the bodyweight of mice fed on an HFD was higher than the change in bodyweight of mice fed with CHOW. The decrease in weight gain between weeks 4 and 5 can be explained by the short period of food deprivation and the stress caused by the intraperitoneal glucose tolerance test. At the end of the experiment, the HFD-I group had gained an additional 58.53%±0.23 and the HFD-C group 61.32%±8.3 of their original bodyweight. In contrast, the groups fed a standard diet had gained significantly less weight (CHOW-I: 118.57%±2.03%, CHOW-C: 111.77%±7.1%). Our DIO mouse model developed obesity, so this phenotype could not be restrained by the oral administration of Ebselen. Prior to the experiments, the mice weighed the same. (HFD-C: 26.57±1.953gr; HFD-I: 27.29±1.286gr; CHOW-C: 26.33gr±1.393; CHOW-I: 27.61±1.242) (**Figure 8B**).

Since bodyweight is influenced not only by food intake but also by the metabolism of the individual and locomotion, it was necessary to observe the energy consumption of the mice. In the case of intraperitoneally injected Ebselen, the drug was able to reduce weight gain in HFD-fed mice without changing the caloric intake, which could be explained by a higher level of physical activity or by a changed metabolism.

In my research, I could not find the exact caloric density of the vehicle. Assuming that all the mice drank the same amount of water, that the amount of vehicle they received in the 9 weeks was 2.025 g for a 30 g mouse or up to 2.9 g for a 43 g mouse, and that the vehicle had 9 kcal/g, the difference in energy intake of the HFD and CHOW mice would be a maximum of 8 kcal, which would account for 1% of the total energy intake. Since there was no weight difference between either the HFD groups or the CHOW groups, and therefore no difference in the applied drug, the intake of calories through the vehicle can be ignored.

Analogously to the previous experiments, the caloric intake of the mice was calculated. The mice which were fed an HFD over the period of 9 weeks (HFD-C: 868.65 kcal ±49.24 HFD-I: 890.03 kcal ±85.42) consumed more calories than the CHOW groups (CHOW-C: 710.24 cal±71.84; CHOW-I 718.253kcal±45.85) (**Figure 8C, D**). This finding is in accordance with the weight gain of the mice.

5.2.3.3 One month of Ebselen treatment prevents glucose resistance in HFD-fed mice, after 2 months of treatment a glucose resistance developed

Even though Ebselen did not prevent the mice from becoming obese, the application of Ebselen did influence glucose homeostasis. In order to monitor the development of glucose tolerance,

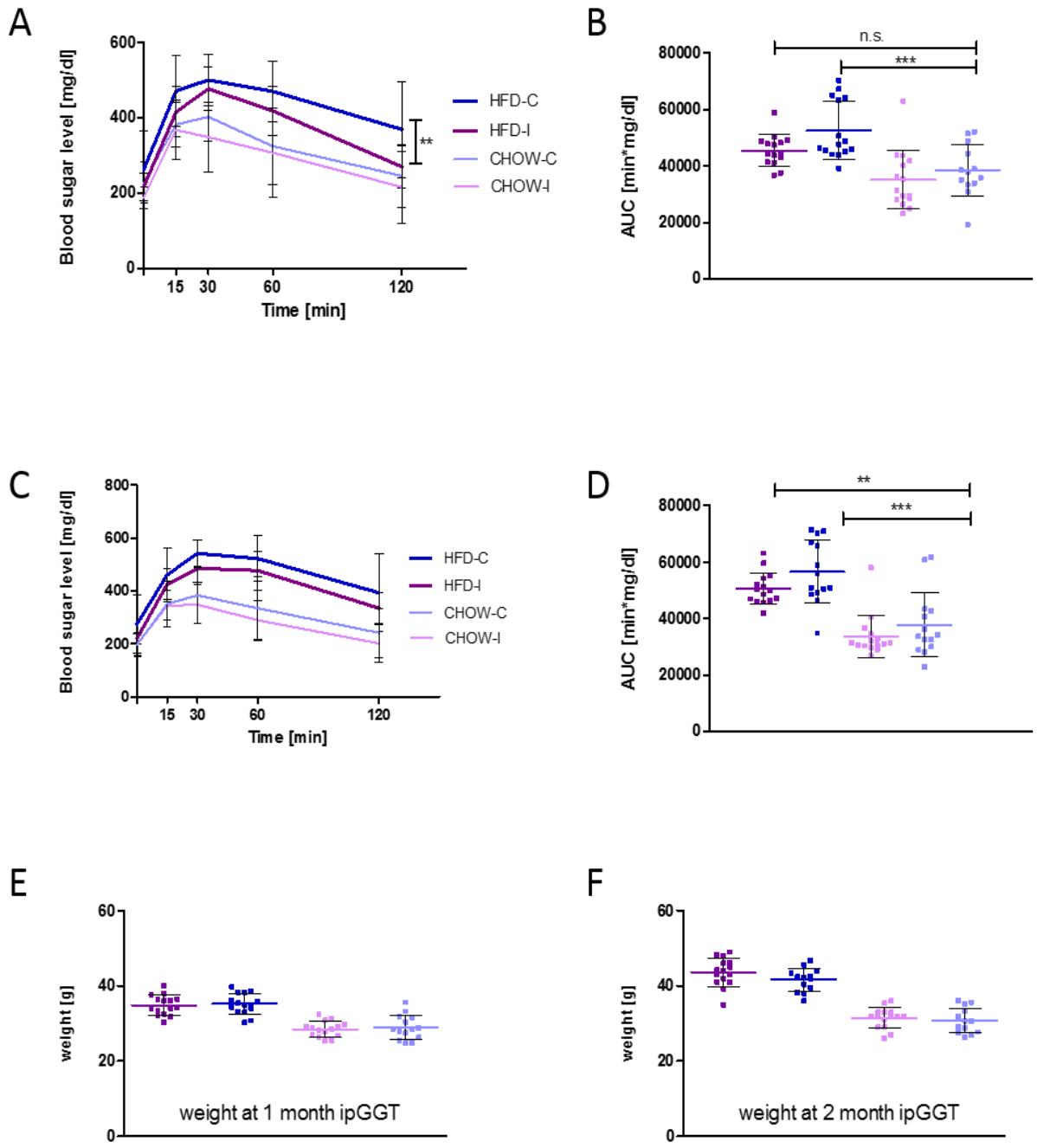


Figure 9: *Ebselen application through drinking water prevents glucose resistance after 1 month, this effect is reversed after 2 months of treatment.* ***A, B*** *ipGTT after 1 month of treatment. HFD vehicle-treated animals have impaired glucose resistance. HFD Ebselen-treated animals are protected from glucose tolerance (N=15).* ***C, D*** *ipGTT after 2 months of treatment. HFD groups developed a glucose resistance.* ***E*** *After 1 month of treatment, HFD led to an obese phenotype (p=***), Ebselen treatment did not influence weight gain (p=ns).* ***F*** *After 2 months of treatment, HFD led to an obese phenotype (p=***), Ebselen treatment did not influence weight gain (p=ns). Each dot represents one measured animal at each time point (N=15), with mean and SD. Statistics: two-way ANOVA or one-way ANOVA with Bonferroni post-test.*

an ipGTT was performed as described after 1 and 2 months. The drinking water with the dissolved Ebselen or vehicle was replaced by pure drinking water during the food deprivation period prior to the ipGTT, since the vehicle is energetic and would lead to false blood glucose levels.

The basal fasting glucose level was similar between the groups (**Figure 9A**). After 15 min, the plasma glucose of the HFD-C was 23.4% higher than the CHOW-C (P=*) and after 30 min, the plasma glucose level was 24.1% (P=**) higher, whereas there was no difference between the HFD-I group and the CHOW-C group. After 60 min, HFD-fed animals had significantly higher plasma glucose levels; Ebselen could not prevent this glucose resistance (HFD-C: 44.8% higher; P=***, HFD-I group: 29%; P=*) At the last time point, after 90 min, the HFD-C group had a 50.1% higher glucose level than the lean control group (P=***), in contrast to the HFD-I group which did not have elevated glucose levels. After 120 min, the glucose level of the HFD-I group (269.6 mg/dl) was significantly reduced compared to the HFD-C group (368.9 mg/dl).

Not surprisingly, the HFD control group showed a significant change in glucose resistance after 4 weeks of treatment (**Figure 9B**). The HFD led to a deterioration in glucose sensitivity, with an increase of 36.4% of the AUC (HFD-C group: 52493 min*(mg/dl) ±10342); CHOW-C: 38475 min*(mg/dl) ±9102, p=***). In contrast, the HFD group treated with Ebselen (AUC=45495 min*(mg/dl) ±5556) did not show a significant increase compared to the CHOW-C group. Between the standard chow-fed mice treated with Ebselen (CHOW-I: 35027 min*(mg/dl) ±10342) and the CHOW-C group there was no difference (p=ns). Four weeks of an HFD led to glucose resistance, which could be averted by the oral administration of Ebselen. This effect is specific for HFD-induced obesity, since no effect could be seen in mice fed the CHOW.

After 8 weeks, the second ipGTT was performed to see the development of glucose tolerance. The effect which had been seen after 4 weeks of treatment had vanished. As in the prior ipGTT, the basal fasting glucose level of all four groups was equal (**Figure 9C**). Fifteen minutes after the glucose injection, the plasma glucose of the HFD-C was 31% higher than the CHOW-C (P=**), while there was no difference between the HFD-I group and the CHOW-C group. After this time point, both HFD groups had a glucose resistance compared to the CHOW-C group. In the lean mice, Ebselen treatment did not influence the glucose levels. As anticipated, the glucose resistance of the obese control group was reinforced after 8 weeks of HFD with an increase of 33.7% in the AUC (HFD-C: 56491 min*(mg/dl) ±10742) compared with the CHOW-C group (37750 min*(mg/dl) ±11465, p=***). Unfortunately, the HFD group treated with Ebselen (AUC=50489 min*(mg/dl), ±5617) had also developed a glucose resistance, thus cancelling out the effect of the Ebselen seen after 1 month (**Figure 9D**). In the HFD control and inhibition group, a decrease in the AUC can be seen. Ebselen does not influence glucose

metabolism in lean mice fed on a CHOW diet (CHOW-I: AUC=35027 min*(mg/dl), ±10342) and the CHOW-C group (p=ns).

Fifteen minutes after the glucose injection, the treatment with Ebselen protects against a higher glucose tolerance, but after 30 min, this effect is reversed. Looking at the whole time course, the overall glucose tolerance is impaired in the HFD-C and HFD-I groups.

There could be several reasons behind the contradictory results that the oral treatment with Ebselen prevented the obese mice from developing a glucose resistance after 1 month, but only slightly affected glucose metabolism after 2 months. Since obesity is associated with glucose resistance, a change in bodyweight could also have been limited temporarily, resulting in a better glucose homeostasis in the HFD-I group compared with the HFD-C group after 1 month of treatment. In order to exclude the possibility that changed bodyweight leads to a change in glucose metabolism, the bodyweight at the time points of the ipGTT was observed more closely (**Figure 9E, F**).

On the day of the first ipGTT, the HFD groups had developed an obese phenotype (HFD-C: 35.22±2.795gr; HFD-I: 34.88±2.650gr; CHOW-C 28.98gr±3.281; CHOW-I 28.48 gr± 2.069; p=***). Ebselen treatment did not influence weight gain in the mice fed an HFD (**Figure 9E**). From this we can conclude that the improved glucose metabolism in mice treated with Ebselen was not due to reduced weight. The second ipGTT showed the same results as after 1 month of treatment (**Figure 9F**): Ebselen treatment did not influence weight change either in HFD-fed mice or in the CHOW-fed mice (HFD-C: 41.66±2.964gr; HFD-I: 43.66±3.75gr; CHOW-C: 30.75gr±3.28; CHOW-I: 31.49±2.96; p=***).

In short, no temporary effects of weight differences in the HFD-fed groups can explain the change in glucose sensitivity after 1 month of treatment with Ebselen.

Time	CHOW-C		HFD-C			HFD-I			CHOW-I		
	Mean	SD	Mean	SD	P'	Mean	SD	P'	Mean	SD	P'
0	228.31	53.53	262.10	103.34	ns	216.38	35.88	ns	188.44	29.02	ns
15	381.77	59.41	471.29	93.08	*	415.42	66.66	ns	367.43	79.91	ns
30	402.94	66.91	500.04	67.78	**	476.8	58.74	ns	348.04	92.2	ns
60	324.38	102.14	469.79	80.41	***	418.31	64.98	*	306.66	118.66	ns
120	245.76	83.68	368.87	127.52	***	269.61	56.35	ns	215.72	95.73	ns
'Compared with CHOW Control (Bonferroni post-test) **Significant at the 0.05 level **Significant at the 0.01 level ***Significant at the 0.001 level*											

Table 3: 1-month glucose tolerance test. Plasma glucose level in mg/dl after glucose injection

Time	CHOW Control	HFD Control	HFD Inhibition	CHOW Inhibition

	Mean	SD	Mean	SD	P'	Mean	SD	P'	Mean	SD	P'
0	196.76	45.67	274.54	109.56	Ns	220.89	19.23	ns	199.9	42.06	ns
15	351.96	85.61	461.22	102.68	**	425.51	57.79	ns	344.63	56.45	ns
30	383.65	103.86	541.7	50.87	***	486.26	53.517	**	349.91	73.72	ns
60	335.01	117.03	522.39	86.958	***	477.04	72.82	***	289.57	74.63	ns
120	242.91	93.05	393.93	146.93	***	334.72	57.91	*	202.67	72.38	ns
'Compared with CHOW Control (Bonferroni post-test) **Significant at the 0.05 level **Significant at the 0.01 level ***Significant at the 0.001level*											

Table 4: 2-month glucose tolerance test. Plasma glucose level in mg/dl after glucose injection

5.2.3.4 The body composition of HFD mice is not changed through the application of Ebselen

Having found no changes in weight gain, but contradictory results in the development of insulin resistance, the body composition of the mice was observed in detail. Lean mass can have a positive effect on glucose metabolism. The treatment with Ebselen did not change the bodyweight of the mice, either when fed a high-fat diet, or when fed the standard diet. Even if the mice weighed the same, there could be a difference in body composition. Since weight gain is mainly due to both an increase in fat and an increase in lean mass, we checked to see whether there were any differences between the groups with H-NMR prior to the final metabolic cage measurements. Both the absolute weight of fat and lean mass and the ratio of lean and fat mass to the total bodyweight were analyzed.

As expected, the groups with a high-fat diet showed an increase in absolute (**Figure 10A**) fat mass in contrast to the CHOW groups (p=***). Through the energy-enriched diet, the mice tripled their fat mass. The application of Ebselen did not change the fat mass between the HFD groups (HFD-I: 18.34gr ±3.569gr; HFD-C: 17. 84 gr ±2.56gr, p=ns) and CHOW-fed mice (CHOW-I: 7.145gr±3.18gr; CHOW-C=6.579gr±3gr). To observe the body composition, the percentage of fat mass to total bodyweight was calculated (**Figure 10B**). Consistently with the absolute fat mass, the percentage of fat mass in the total bodyweight was doubled (HFD-I: 43.23% ±10.17%; HFD-C: 40.82% ±2.58%; CHOW-I: 21.35% ±9.28%; CHOW-C: 19.84% ± 6.65%, P=***). There was no difference between either the HFD-I and HFD-C groups or the CHOW-I and CHOW-C groups.

To sum up, an HFD led to an increase in total fat mass and in the percentage of fat mass of the total bodyweight; Ebselen did not have any effect on the fat mass.

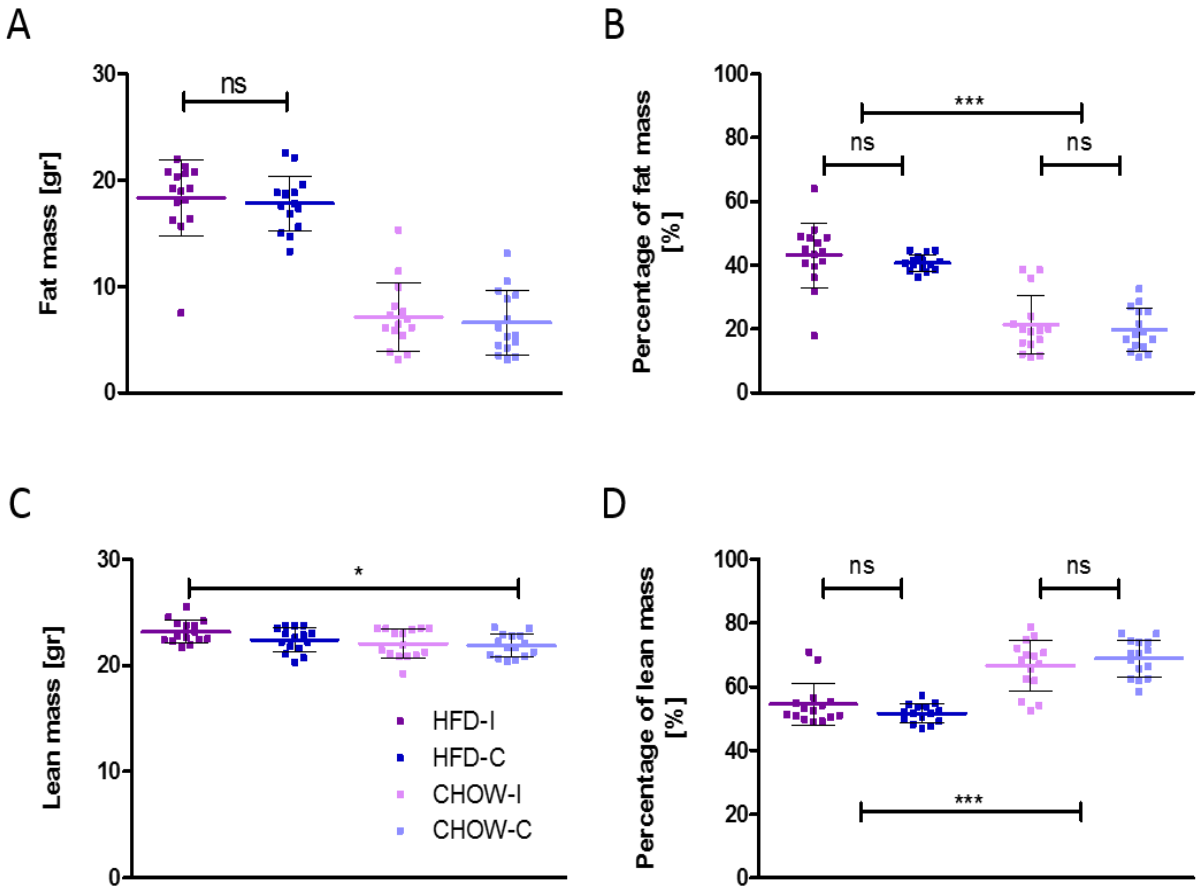
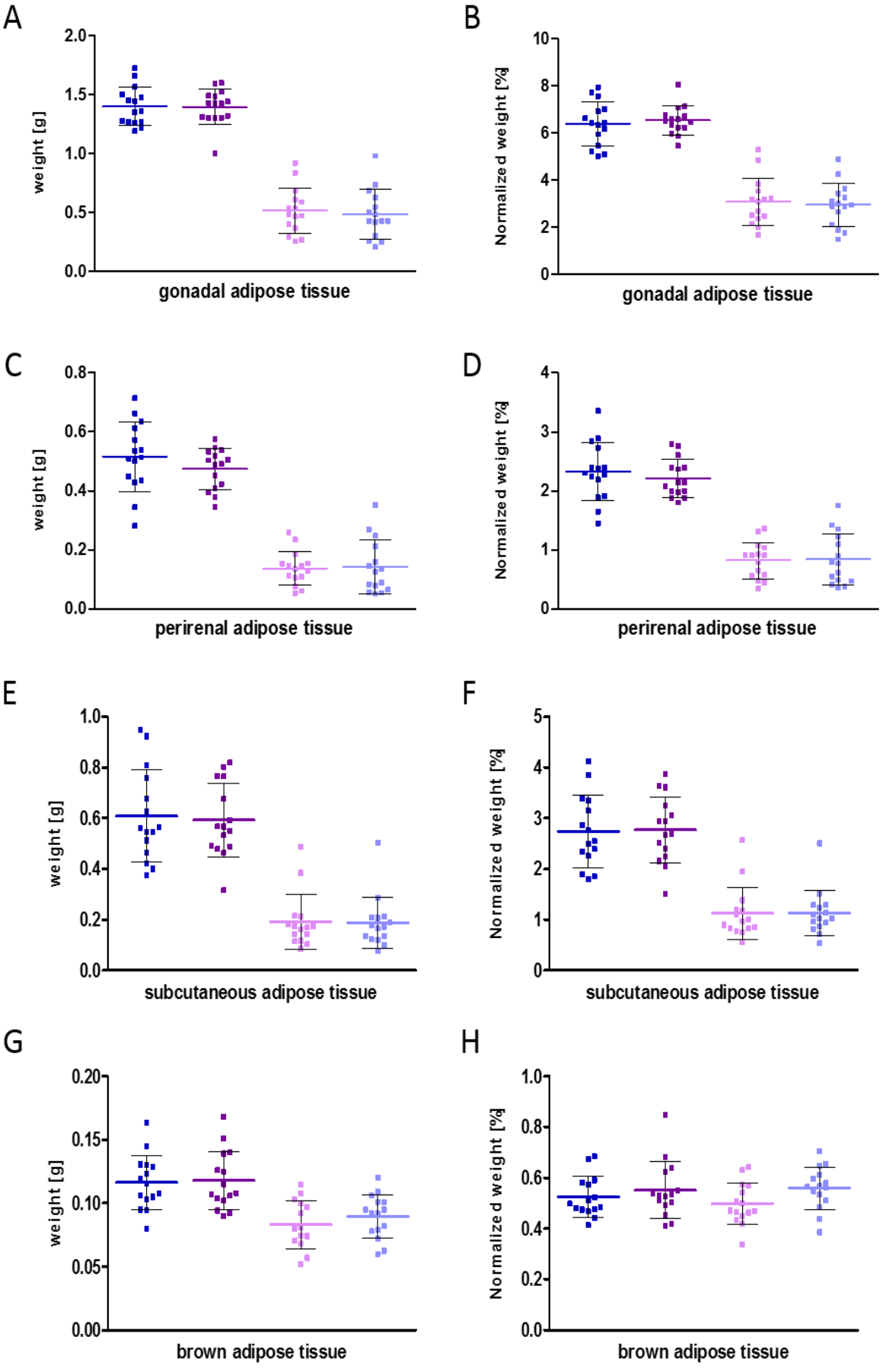


Figure 10: *General body composition of mice is unchanged after 2 months of oral administration of Ebselen in the HFD-IO.* ***A*** *Absolute fat mass measured by H-NMR, HFD caused growth of total fat mass, Ebselen does not influence weight of AT.* ***B*** *Percentual fat mass of total bodyweight, increase in bodyweight is due to increase of fat mass, Ebselen does not change the fat composition.* ***C*** *Lean mass measured by H-NMR, HFD does not have an effect on absolute fat mass, treatment of Ebselen leads to an increase of total lean mass.* ***D*** *Percentual lean mass of total bodyweight; Ebselen does not influence the percentual lean mass. Each dot represents one measured animal at each time point (N=15), with mean and SD. Statistics: one-way ANOVA with Bonferroni post-test.*

Figure 11: *(next page) Absolute and relative mass of different fat compartments is unchanged after 2 months of oral administration of Ebselen.* ***A,B*** *Absolute(**A**) and relative (**B**) mass of gonadal adipose tissue is increased by HFD (p=***). Ebselen administration does not influence gonadal adipose tissue.* ***C,D*** *Absolute (**C**) and relative (**D**) mass of perirenal adipose tissue is increased by HFD (p=***). Ebselen administration does not influence perirenal adipose tissue.* ***E,F*** *Absolute (**E**) and relative (**F**) mass of subcutaneous adipose tissue is increased by HFD (p=***). Ebselen does not have an effect.* ***G,H*** *Absolute(**G**) mass of brown adipose tissue is increased by HFD (p=**), Ebselen administration does not have an effect on the weight of BAT. Normalized weight of BAT to BW of mice (**H**) is neither changed by HFD nor by Ebselen. Each dot represents one measured animal at each time point (N=15), with mean and SD. Statistics: one-way ANOVA with Bonferroni post-test.*


A
weight [g]
2.0
1.5
1.0
0.5
0.0
gonadal adipose tissue
B
Normalized weight [%]
10
8
6
4
2
0
gonadal adipose tissue
C
weight [g]
0.8
0.6
0.4
0.2
0.0
perirenal adipose tissue
D
Normalized weight [%]
4
3
2
1
0
perirenal adipose tissue
E
weight [g]
1.0
0.8
0.6
0.4
0.2
0.0
subcutaneous adipose tissue
F
Normalized weight [%]
5
4
3
2
1
0
subcutaneous adipose tissue
G
weight [g]
0.20
0.15
0.10
0.05
0.00
brown adipose tissue
H
Normalized weight [%]
1.0
0.8
0.6
0.4
0.2
0.0
brown adipose tissue

In contrast to these findings, the absolute weight of the lean mass of the HFD mice who drank Ebselen increased by 5.85% in comparison to the lean control group (HFD-I: 23.16 ± 1.08g; CHOW-C: 21.88g ±1.08, p=* (**Figure 10C**), while the lean mass was not increased by the HFD alone (HFD-C vs. CHOW-C: p=ns). In the lean control group, Ebselen did not influence the lean mass.

Administering Ebselen to the mice was not the only factor leading to the change in the lean mass, since the lean mass of the HFD-I vs. HFD-C group and the CHOW-I vs. CHOW-C group was identical. When comparing the HFD-I group to the CHOW-I group, no increase of lean mass was seen. Since there is no significant difference between these groups, the impact could be a combination of the HFD and the Ebselen treatment (CHOW-I: 22.06 g ±1.36).

The relative lean mass did not show any surprising results. It was lower in the groups fed a high-fat diet, since they have an increased amount of fat mass (HFD-I: 54.50%±6.59%; HFD-C: 51.69%±2.9%; CHOW-I 66.63%±7.98%; CHOW-C: 68.92%±5.76%, P=***). There was no difference between either the HFD-I and HFD-C groups or the CHOW-I and CHOW-C groups.

As well as the general distribution of lean mass and AT in the mice, we closely examined the different fat compartments of the mice. In addition to lean mass, the distribution of fat pads over the body also interferes with glucose resistance and the metabolic syndrome. A difference in glucose metabolism between the HFD-C and HFD-I groups with similar fat mass could be due to a difference in body fat distribution. A mouse has different fat compartments. The most important metabolic differentiation is between visceral (gonadal and perirenal) fat and subcutaneous fat, since visceral fat correlates with an abnormal metabolic profile [68] and is a better predictor of the metabolic syndrome than total fat mass [69, 70]. Epidemiological studies have shown a link between central obesity and insulin resistance and mortality in humans. It is also recognized that an increased amount of lower-body fat is related to a decrease in the risk of metabolic complications. The expression and release of adipokines from adipose tissue are different in specific depot sites (omental, mesenteric, subcutaneous), with visceral fat having greater macrophage infiltration and therefore greater expression of pro-inflammatory- and lower expression of anti-inflammatory adipokines than subcutaneous fat [71]. The NLRP3 inflammasome also seems to have a distinctive effect on VAT and SAT, since the genetic ablation of NLRP3 is associated with a reduction in fat cell size in VAT but not in SAT [43].

Different fat compartments, including gonadal fat, perirenal fat, subcutaneous and BAT were, therefore, manually collected from one body half and weighed. Since it is not possible to collect all the subcutaneous fat, an accurately defined part of one side of the carcass was taken. As brown adipose tissue can exist in various parts of the body, I chose half of the brown adipose tissue (BAT) between the scapulas as an exemplary mass for the BAT.

In the obese groups each compartment, excluding BAT, weighed approximately 1.8 times more than in the CHOW-fed groups (HFD-C, HFD-I vs CHOW-C: p=***, **Figure 11A, C, E**). The BAT of the HFD groups was approximately 1.4 times greater than that of the CHOW-fed groups (HFD-C, HFD-I vs CHOW-C: p=**) (**Figure 11G**). The administration of Ebselen did not change the size of the different fat compartments, but the HFD led to an absolute increase of all fat compartments.

Analogous to the H-NMR, the relative fat mass, normalized to body weight, was calculated. In both HFD groups, the weight gain led to an increase in gonadal, perirenal and subcutaneous adipose tissue (HFD-C vs CHOW-C: p=***; HFD-I vs CHOW-C: p=***) (**Figure 11B, D, F**). As expected, the relative fat mass of BAT is not changed by an HFD, since brown adipose tissue does not act as the main storage depot for excess energy (**Figure 11H**). The distribution of fat compartments was not changed by the administration of Ebselen (HFD-C vs HFD-I: p=ns, CHOW-C vs CHOW-I: p=ns).

In sum, the application of Ebselen did not influence fat mass, whether total fat mass or in the fat compartments.

5.2.3.5 Ebselen treatment does not change the movement and energy expenditure of mice in DIO

After one month of treatment at the latest, the mice showed an improved glucose metabolism. Two important metabolic parameters, locomotion and energy expenditure, were therefore measured in the metabolic cages. In addition, as a behavioral change through higher physical activity could explain the slight increase of lean mass in the mice, we wanted to exclude any change in locomotion as the reason for this increase.

The mice were measured in the metabolic cages both prior to and after the experiments. The metabolic cages count the movements in the x- and y-axis in one measurement period (33 min). Since only 10 metabolic cages were available, it was not possible to measure all the animals on

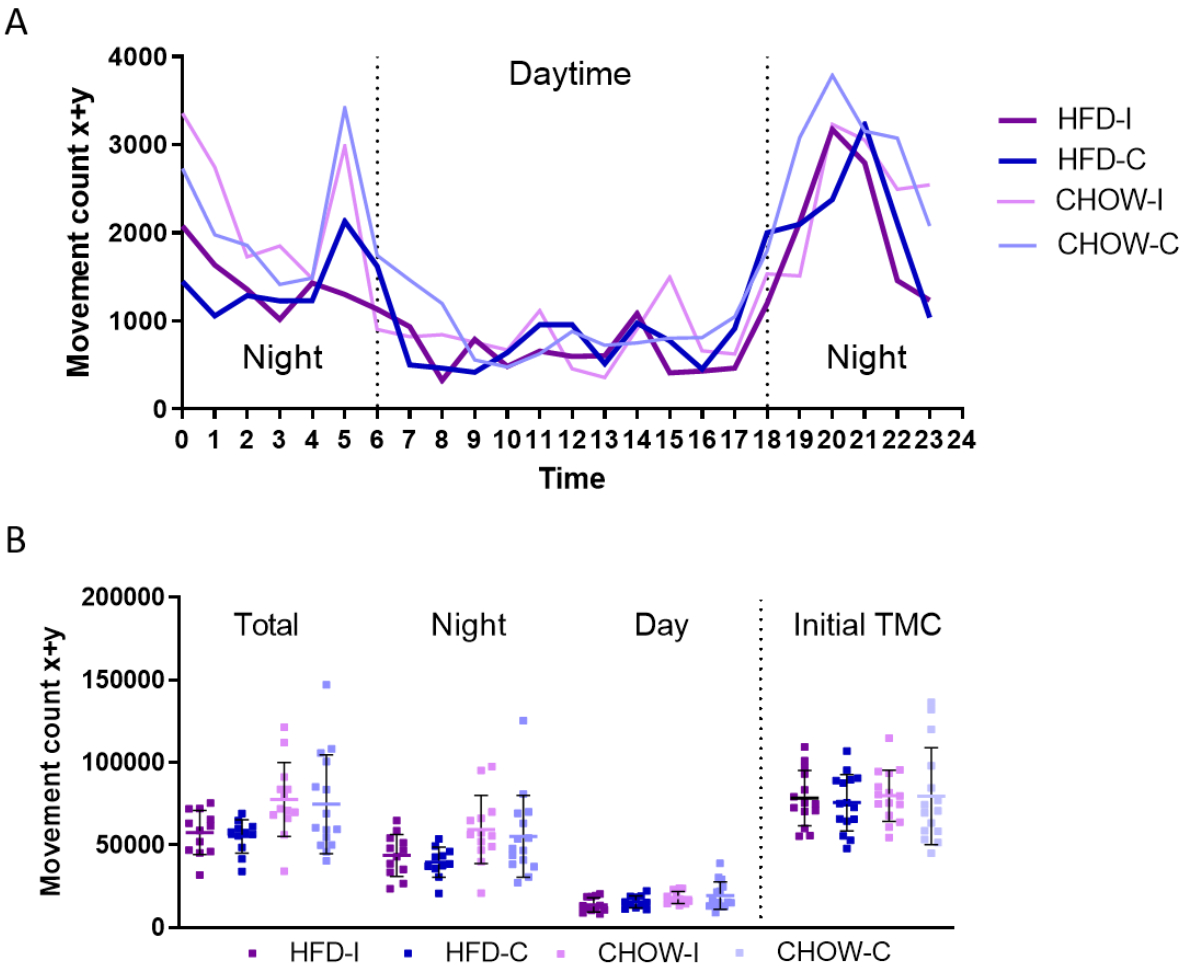


Figure 12: Locomotion of mice is unchanged after 2 months of oral Ebselen treatment. **A** Mice show a normal day/night movement pattern. **B** HFD does not change TMC, daytime or nighttime movement. Ebselen does not influence movement of the mice. Prior to the experiment (initial TMC) movement of the mice was equal. Each dot represents the value of the movement of one measured animal (N=15), with mean and SD. Statistics: one-way ANOVA with Bonferroni post-test.

the same day. Because of this, different time points were taken on the different days. For comparability purposes, I calculated the mean movement of each hour and graphed the development of the course over the day. This provided indications of similar movement patterns. To quantify changes, I calculated the total movement counts per day (TMC). For each animal, this meant 44 summands per day. This sum was further subdivided into daytime movement (6 a.m. to 6 p.m.) and nighttime locomotion (6 p.m. to 6 a.m.). For these calculations, all daytime measurements and all nighttime measurements were added together. Both groups were the sum of 22 time points.

The movement of the mice was relatively low during the light phase and increased as the dark phase began at 6 p.m., indicating that all groups of mice displayed a normal circadian rhythm (**Figure 12A**). The total movement was the same in all the groups. Ebselen treatment did not

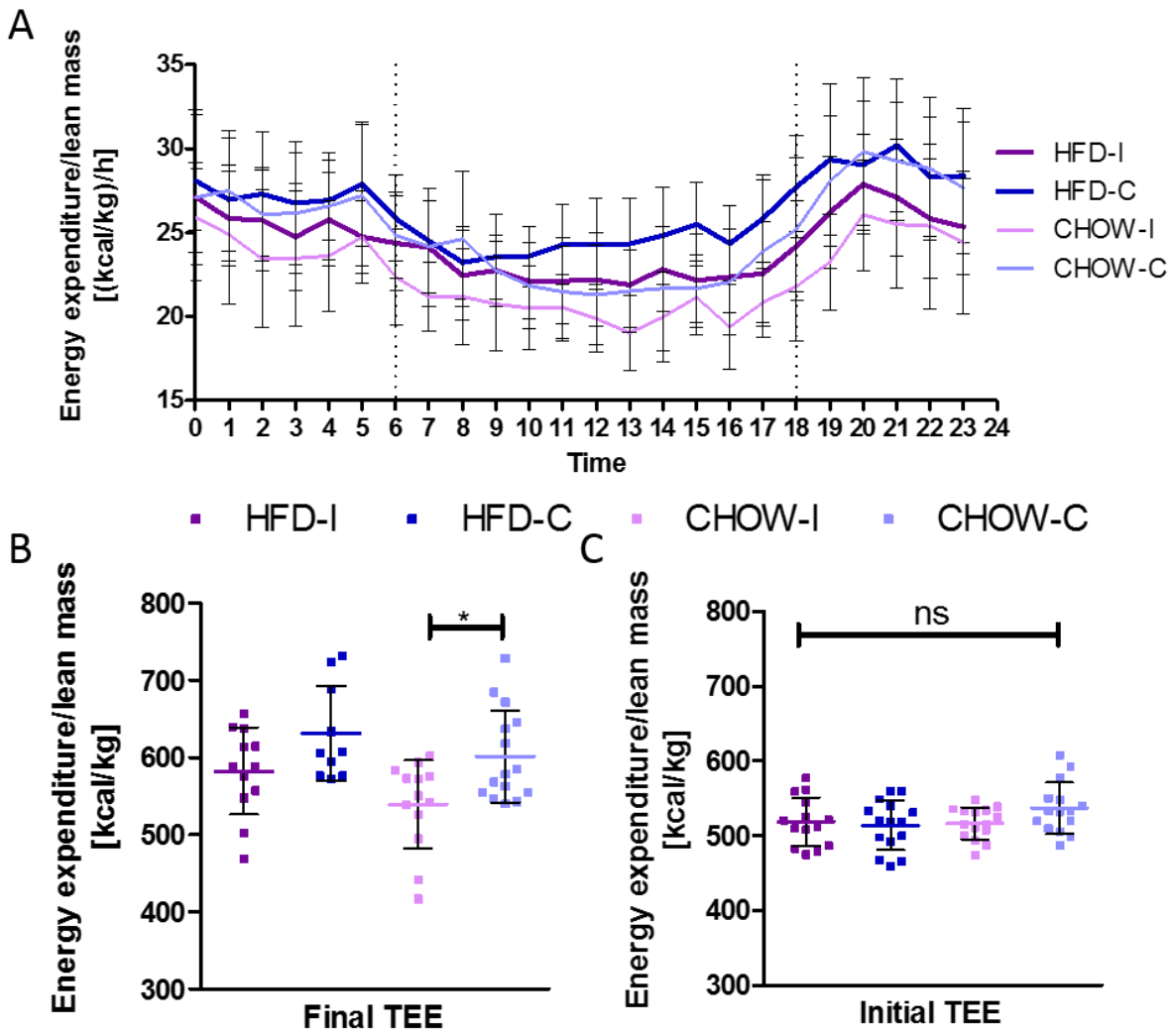


Figure 13: *Energy expenditure in HFD-IO mice is not changed by Ebselen. Ebselen reduces energy expenditure in lean mice when applied orally.* ***A*** *Energy Expenditure profiles after 8 weeks of HFD and oral Ebselen treatment, mice show a normal day and night pattern.* ***B*** *Total energy expenditure of mice, HFD does not change energy expenditure in mice. Oral Ebselen treatment does not change TEE in obese mice; in lean subjects, Ebselen decreases TEE in mice.* ***C*** *Total energy expenditure before the experiment. Prior to the experiment, all groups had equal energy expenditure. Each dot represents one measured animal or the mean of EE per hour of each group with mean and SD. Statistics: one-way ANOVA with Bonferroni post-test.*

influence the locomotion in HFD or CHOW-fed mice (HFD-C vs HFD-I ns; CHOW-C vs CHOW-I: ns). When subdividing the movement into sleeping time (daytime) and waking time (nighttime) there was still no difference between lean and obese mice. Prior to the experiment and at the end of the experiment, the mice were measured in the metabolic cages. The initial measurements did not show a difference between the groups (**Figure 12B**).

The Labmaster measured the oxygen and carbon dioxide concentration in every cage. Using these two measurements, the respiratory expiratory rate was calculated and then the energy

expenditure (kcal/kg/h) could be calculated adjusted to lean mass. As explained before, not all animals could be measured on the same day. Therefore, the mean for each hour was calculated and then graphed. In addition, the TEE, the sum of the actual EE per hour of the day was calculated.

In addition to the locomotion, the energy expenditure of the mice was calculated. The improved insulin sensitivity observed in HFD mice orally treated with Ebselen after 1 month of treatment could be linked to differences in their basic metabolic parameters. To assess this, all groups were subjected to indirect calorimetric analysis using metabolic cages.

The graph of energy expenditure over the day shows the expected day and night patterns of the mice: reduced energy expenditure during the day and an increase during the night, when the mice are active (**Figure 13A**). The total energy expenditure was not changed by HFD feeding over 2 months (**Figure 13B**). In the obese mice, the Ebselen treatment did not change the energy expenditure. Surprisingly, the energy expenditure was reduced by Ebselen treatment in the control chow-fed mice (CHOW-C vs CHOW-I: p=*). The administration of Ebselen led to a decrease of 10.12% over the untreated group. In the initial measurements prior to the start of the experiment, the TEE of all groups was equal (**Figure 13C**). The decrease of TEE in lean mice surprised us and we do not have an explanation for this change.

5.2.3.6 Ebselen does not change IL-1β protein levels in blood serum and VAT

Even though the treatment with Ebselen did not influence the metabolic phenotype of the mice, there should be a decrease of IL-1β in accordance with the previously described *in vitro* experiments (see Figures 3, 4).

Directly after sacrificing the mice, blood was drawn directly from the heart and then centrifuged. Later, the blood serum was analyzed for IL-1β levels using ELISA. Little IL-1β could be detected in the serum (**Figure 13A**), since it was only possible to attain the minimal detectable amount of 8 pg/ml from 6 out of 36 mice (HFD-I: N=8, HFD-C: n=10, CHOW-I: n=7, CHOW-C: n=11). The HFD did not induce an increase in IL-1β level independently of Ebselen treatment. As no obese phenotype could be detected between the HFD-C and CHOW-C groups in IL-1β levels, the Ebselen treatment was not able to block the obese phenotype. In the lean groups, the Ebselen application did not influence the IL-1β levels (HFD-I: 2.89 pg/ml±5.483; HFD-C: 3.671pg/ml±7.826; CHOW-I: 7.649pg/ml±14.66; CHOW-C:

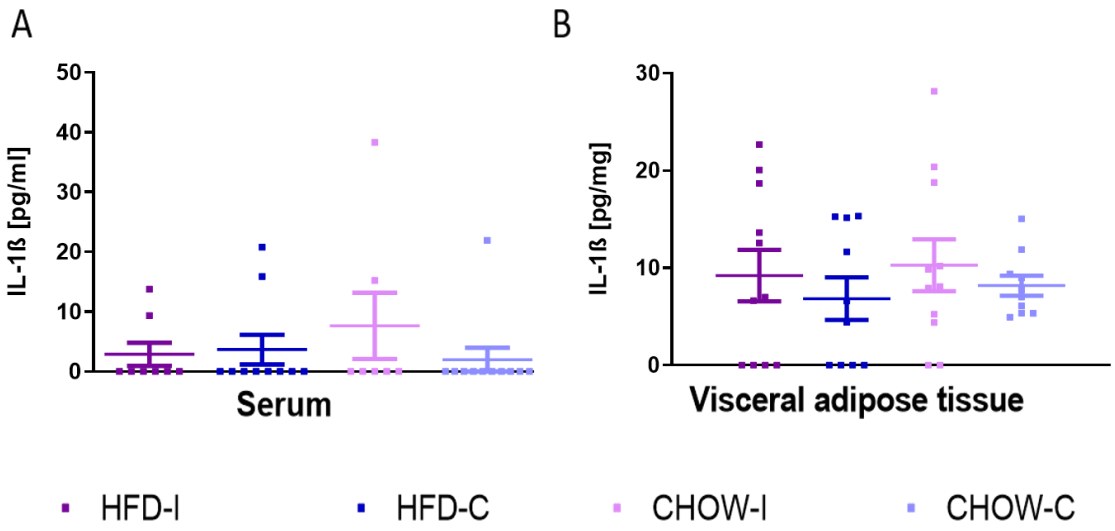


***Figure 14**: IL-1β levels are not increased in the VAT and serum of DIO mice after 2 months of HFD. **A** IL-1β protein levels in serum are not increased in the HFDIO-mice, nor reduced upon Ebselen treatment. **B** IL-1β protein levels are not increased in obese mice compared to lean animals. Therefore, Ebselen treatment was not able to reverse any effect of the HFD. Each dot represents one measured animal, with mean and SD. Statistics: one-way ANOVA with Bonferroni post-test.*

1.994pg/ml±6.613). To conclude, our DIO obesity model did not show increased IL-1β levels in the blood after 10 weeks of an HFD.

Since the Ebselen treatment did not change IL-1β levels in the blood serum, the VAT was analyzed for IL-1β content using ELISA. The VAT were processed via homogenization in a RIPA lysis buffer. The protein was extracted and then the protein concentration was measured using a BCA assay. Next, the amount of IL-1β in 1mg protein was calculated.

Surprisingly, the HFD did not induce an increased IL-1β level at this time point (HFD-C=6.846pg/mg protein±6.902; CHOW-C: 8.179pg/mg protein±3.263, p=ns). The Ebselen treatment also did not change the amount of IL-1β, either in the HFD groups (HFD-I: 9.210pg/mg protein±8.780. p=ns) or in the CHOW groups (CHOW-I: 10.28pg/mg protein ±8.813. p=ns).

To recapitulate, the application of Ebselen through drinking water did not have any influence on IL-1β levels in the blood serum and gonadal fat pads after 2 months. More importantly, the HFD did not induce elevated IL-1β levels.

6 Discussion

Unpublished information revealed that Ebselen is a specific NLRP3 inflammasome inhibitor. In this thesis, the effect of Ebselen *in vitro* and in the context of HFD-induced obesity has been characterized. The aim of my project was to test the efficacy of Ebselen *in vitro* (**Aim 1**) and *in vivo* to assess the effects of inflammasome inhibition on high-fat diet-induced obesity and metabolic syndrome in wild-type mice, focusing on the development of obesity and insulin resistance (**Aim 1**). To evaluate the first aim, the potency of Ebselen was tested *in vitro* on two important cell types of the innate immune system, peritoneal macrophages and microglia. These cells were manipulated to reverse their impaired phenotype into that of a more functional one by inhibition of the NLRP3 inflammasome by Ebselen. After finding positive results in this type of cell, the second aim of the study was pursued, to test the efficacy of Ebselen as an NLRP3 inflammasome inhibitor *in vitro*.

6.1 Aim 1: Ebselen inhibits the release of IL-β in activated peritoneal macrophages and microglia

To study the effect of Ebselen on peritoneal macrophages, a peritoneal macrophage cell culture was established in our laboratory. Following this, the inhibitory potential of Ebselen on peritoneal macrophages and microglia was studied. As described, two steps are required to activate the NLRP3 inflammasome, in my experiment a priming signal from LPS-stimulated Toll-like receptor 4 and an activation signal from another stimulus, such as ATP. The first step induces the expression of NLRP3 and pro-IL-1β, and the latter controls caspase-1 activation, which leads to splicing of pro-IL-1β and the release of this cytokine [72]. For the peritoneal macrophages, we used a widely-used and confirmed protocol to induce NLRP3 activation with LPS and ATP [73, 74].

Following this protocol, we induced IL-1β secretion. Incubation of peritoneal macrophages with Ebselen significantly inhibited IL-1β secretion induced by LPS+ATP. It made no difference whether Ebselen was added simultaneously with LPS and ATP or was just added with ATP. In our laboratory, Natalie Drost used a comparable setup and confirmed my results in peritoneal macrophages [75]. Additionally, she showed the tolerability of Ebselen up to a concentration of 100 μM with a viability assay.

For microglia, we chose another time course. Stimulation of the microglia cells with LPS and ATP together with 100 μM Ebselen during the LPS and ATP application showed a suppression of inflammasome activation in microglia, measured by IL-1β secretion, that was similar to the peritoneal macrophages. Furthermore, adding Ebselen only with ATP was able to inhibit IL-1β secretion.

We interpreted the reduction of IL-1 β secretion when only applying Ebselen simultaneously with ATP as the ability of Ebselen to inhibit the second activation step of the NLRP3 inflammasome. We are aware that this is not yet proven, and further analysis is required to provide conclusive evidence.

IL-1β is one of the cytokines produced and released by activated NLRP3 inflammasomes. IL-1β is a major pro-inflammatory cytokine, mainly produced by macrophages and found in an increased concentration in adipose tissue in obese subjects [76]. Therefore, as proof of principle, I measured IL-1β in the supernatant as indirect proof of inflammasome activation. Our experiment showed that Ebselen was able to inhibit IL-1β secretion in peritoneal macrophages and microglia. This result is in line with the assumed pharmaceutical mechanism of action, but does not prove that Ebselen is a NLRP3 inhibitor, since we did not measure any NLRP3 inflammasome components (such as ASC) or the proform of the released cytokines.

Our result, that adding Ebselen only to ATP is sufficient to suppress IL-β release, is contradicted in a study by Jabaut et al., in which it is suggested that in macrophages, Ebselen inhibits NLRP3 inflammasome priming (“signal 1”) but not NLRP3 inflammasome activation (“signal 2”)[76]. In their setup, the peritoneal macrophages were only stimulated by serum amyloid A for an extended time (24 h). Adding Ebselen prior to serum amyloid alpha inhibited IL-1β secretion, but when added after 8h, it had no effect. The discrepancy between their results and ours could be due to the different concentration of Ebselen, using only one stimulant and a different time course in the experiment. An analysis of different components of the NLRP3 inflammasome, pro-IL1β and pro-IL18 would help to confirm our hypothesis.

As explained, chronic inflammation in obesity is not limited to peripheral organs but also affects the CNS. This inflammation can originate from peripheral immune cells, such as macrophages, or from microglia [77]. Therefore, it was very important to test the ability of Ebselen to inhibit the NLRP3 inflammasome or IL-1β secretion in microglia in order to indicate that Ebselen could also inhibit central meta-inflammation in the context of an HFD and obesity. This

hypothesis is supported by various studies which have shown that microglia are primed by exposure to a high-fat diet. Erion et al. demonstrated that forebrain microglia of leptin-receptor deficient *db/db* mice produce increased amounts of IL-1β [78] and that central administration of IL-1RA averted memory impairment caused by an HFD [79].

Some pharmaceuticals which have recently been shown to inhibit NLRP3 activation are also NF-kB inhibitors, therefore inhibiting the priming steps [80-82]. It would have been interesting to examine whether Ebselen also inhibits NF-kB. This could be done by applying Ebselen to the LPS administration to inhibit the priming and then leaving it out during the activation step. Even if Ebselen does not inhibit the NLRP3 inflammasome, just reducing IL-1β, one of the main pro-inflammatory cytokines leading to meta-inflammation in obesity, would be a major success. To name but a few of the effects of IL-1β in the context of obesity and insulin resistance, IL-1β causes decreased expression of glucose transporter GLUT4, IRS1, p85 and as a result, suppresses insulin signal transduction. Furthermore, this cytokine has been shown to induce apoptosis in pancreatic β-cells [83]. Ebselen would be a useful pharmaceutical for inhibiting IL-1β secretion peripherally and centrally, since in contrast to other IL-β blockers, such as Anakinra, it crosses the blood brain barrier and reached the CNS when it was used in mice [60]. This is another advantage of Ebselen over other drugs.

To sum up: Ebselen inhibits IL-ß release into the cytosol in peritoneal macrophages and microglia after the stimulation of cells with LPS and ATP. We suggest that this effect could be due to inflammasome inhibition, since IL-1β secretion was also reduced when Ebselen was only added simultaneously with ATP. We therefore concluded that Ebselen is effective in reducing IL-1β secretion *in vivo* in the context of HFD-induced obesity and continued with the *in vivo* experiments.

6.2 Aim 2: Ebselen in the context of HFD-IO *in vivo*

6.2.1 Administration of Ebselen through intraperitoneal injections shows promising results, but the clinical use of this drug cannot be approved due to its side effects

For the application of Ebselen *in vivo*, the HFD-IO-mouse model was used to observe whether the development of obesity itself could be influenced and reduced, and whether impaired glucose metabolism could be improved. Fully-grown, 90-day-old male BL6 mice were fed a high-fat diet. To interfere with the start of meta-inflammation caused by the high-fat diet, the treatment with Ebselen was started simultaneously with the change of food. The mice were

intraperitoneally injected with 10 mg/kg Ebselen 3 times a week. At this dosage level, it was shown that 10 mg/kg Ebselen injected intraperitoneally could cross the brain blood barrier and reach the brain [60].

Surprisingly, 55.5% of the Ebselen-treated HFD-fed mice and 30% of the Ebselen-treated CHOW-fed mice had died by day 86, whereas none of the vehicle-injected animals had died. Natalia Drost, who also worked in our laboratory on the inhibition of the NLRP3 inflammasome, albeit in the context of Alzheimer's disease, observed similar toxicity for the Ebselen treatment [76]. The APP+ mice, which were also treated with 10 mg/kg Ebselen 3 times a week, showed a 50% reduction in survival rate after 70 days of treatment. She analyzed blood from these mice after interrupting the Ebselen treatment for 15 days. At that time point, levels of C-reactive protein (CRP), an indicator of systemic inflammation, were not elevated. There was no increase in lymphocyte numbers, and there were no differences in liver and kidney parameters between Ebselen and vehicle-treated animals.

We both detected a swollen belly and signs of Ebselen precipitation in the peritoneum of the deceased mice. An examination of the peritoneum indicated a local peritonitis as the cause of death in these animals. In the control group, treated with the vehicle only, no mice died during the experiment. The doses of 25% ß-cyclodextrin are tolerable, with only minor side effects. Even though intraperitoneal injection can also lead to peritonitis and damage to organs of the abdomen [88], we exclude this as the reason for the death of the mice, since all groups, including vehicle groups, were injected intraperitoneally. Since CHOW-I and HFD-I groups were affected and there was no significant difference in mortality between these groups, the mortality rate was not connected to the diet. A postmortem autopsy of one of the deceased mice showed signs of local peritonitis. We concluded that Ebselen is an irritant which may lead to ileus and peritonitis in rodents, with subsequent adhesions when injected intraperitoneally. Ebselen is listed as a safe compound. The time courses of other published experiments showed a shorter duration of treatment with intraperitoneally injected Ebselen. In most experiments with several injections over a number of weeks, other methods of administration were used. Other groups have probably made similar observations which have not yet been published.

Despite the mortality caused by the intraperitoneal injections of Ebselen, we decided to continue with our analysis of the data collected concerning physiological changes in the mice.

After only 4 weeks, the feeding of high-fat food led to impaired glucose sensitivity in the HFD-C group. This effect was impeded by the intraperitoneal injection of Ebselen, and glucose sensitivity was improved by the application of Ebselen in the DIO model. The improved glucose sensitivity was specific for the DIO model, since in mice fed a standard diet, the Ebselen treatment did not alter glucose sensitivity. Since diet influenced the glucose metabolism in mice injected with Ebselen, the application of Ebselen also had an effect on the mice fed with a standard diet.

Striking differences in bodyweight gain between treatment groups were observed over the course of this study. The treatment with Ebselen prevented the mice from becoming obese after only 1 week. This effect increased during the experiment. The reduction of 27% of bodyweight is comparable to the genetic NLRP3 knockout [85]. As expected, Ebselen did not reduce the weight of those mice fed a standard diet. The weekly caloric food intake and the cumulative caloric food intake were not changed by the Ebselen treatment, which excludes the change in weight gain being the result of less caloric intake. Thus, while the treatment with Ebselen did not alter caloric intake in HFD-fed mice but prevented them from becoming obese, intraperitoneal injections of Ebselen altered metabolism so that less weight was gained per calorie consumed. Even though this could lead to the assumption that the inhibition of the NLRP3 inflammasome was effective, the peritonitis and the associated inflammation and illness of the mice could also have led to a decrease in bodyweight. Furthermore, the scar tissue resulting from local peritonitis in the mice could have led to restricted intraperitoneal absorption of glucose. We cannot say whether the altered metabolism of the mice was due to illness or inhibition of the NRP3 inflammasome. My colleague Natalia Drost, who applied Ebselen intraperitoneally in an Alzheimer's mouse model, measured a reduced plaque number [75], which was probably due to the inhibition of the inflammasome. In her Alzheimer's disease mouse model, the local peritonitis probably did not have a great influence on the CNS, so her results suggest that the changes were due to the pharmacological potency of Ebselen.

Even though the toxic effect of Ebselen seems to be a local effect, I did not analyze the i.p. treated mice further, since the peritoneum is a metabolically active organ and the adhesion of the small and big intestine could affect food intake and absorption.

Therefore, we are not able to say whether the prevention of obesity was due to an inflammation leading to a higher calorie usage or the desired effect of Ebselen, namely inhibiting the NLRP3 inflammasome.

6.2.2 Oral administration of Ebselen in drinking water does not have side effects on mice, but no strong effect on the metabolism of the mice was observed

Since the cause of death had to be due to either the method of application of Ebselen or the drug Ebselen itself, we looked more closely at published studies where Ebselen had been used. In other studies, treatment of mice with 10 mg/kg Ebselen over 154 days was well tolerated. This is supported by the fact that Ebselen is used in clinical trials and considered non-toxic. In these studies, Ebselen was applied orally through oral gavage [59, 66, 86] and also administered dissolved in water [63]. All the studies where Ebselen was applied through intraperitoneal injections had shorter time courses (approximately 5 days) [87, 88]. This research did not mention any side effects of Ebselen. Regrettably, there is no published data available identifying Ebselen as having a local toxic effect in the peritoneum when used for an extended period of time.

To check whether the improved weight and glucose tolerance was due to the peritonitis or the result of the pharmaceutical inhibition of the NLRP3 inflammasome in our first *in vivo* experiment, the experiment was repeated, but instead of administering Ebselen through intraperitoneal injections, the Ebselen was dissolved in drinking water. We decided against oral gavage to reduce animal stress and suffering.

Even though Ebselen dissolved in drinking water had been administered to humans, we had to show that Ebselen is stable in solution for a week when exposed to light and kept at room temperature. After showing that this did not impair the efficacy of Ebselen, we began the experiment.

The water with Ebselen was measured and changed every week. The water intake was measured in the metabolic cages before giving the mice Ebselen or the HFD. At that time point, the mice were drinking approximately 3.8 ml water each day, which was comparable to the weekly average water intake. Furthermore, during the experiment, the HFD-C consumed less water than the animals on CHOW-I. This difference is probably due to HFD pellets having a higher water content than CHOW pellets. Similar effects have been reported in other publications [89]. As expected, there were no fatal casualties using oral Ebselen application and no side effects were seen. Natalia Drost, who used the same application method in an Alzheimer's mouse model to avert the toxicity of intraperitoneal injections of Ebselen, also saw no elevation of CRP or liver enzymes in mice treated orally with Ebselen [75].

Unexpectedly, Ebselen did not reduce weight gain and the caloric intake was not changed. After 1 month of treatment, Ebselen prevented HFD-induced impaired glucose homeostasis, underlining the pharmaceutical potency of Ebselen. One month later, after 8 weeks, this effect had diminished and oral application of Ebselen did not protect the mice from glucose resistance. It led to a decrease in glucose levels in the serum at only one time point in the ipGTT Ebselen application. In addition, Ebselen did not affect glucose homeostasis in chow-fed controls, which suggests specific inhibition of NLRP3 inflammasome activation in diet-induced obesity.

There could be different reasons behind these conflicting results. Similar tendencies have been observed in genetic knockout mice, where in early stages of obesity, the genetic elimination of the NLRP3 inflammasome provides a higher protection against HFD-induced insulin resistance [43]. The reason for this has not yet been explained. However, other studies have shown further contradictory results. One study showed mice lacking caspase-1 to be more obese than the controls [90]. It was speculated that the cause of this change was a variation in gut microbiota in mice raised in different animal facilities. The intestinal microbiome has been shown to play an important role in metabolic diseases [91]. Additionally, IL-18, one of the products of inflammasome activation, has been shown to protect against obesity and insulin resistance, since mice lacking IL-18 are more obese and have hyperphagia and insulin resistance [92]. This could be another reason why the results of our experiments were inconclusive.

Since the oral application of Ebselen did not change bodyweight in mice fed an HFD, we examined the weight of the mice on the days of the ipGTT, since temporary changes in bodyweight could affect the glucose homeostasis. Ebselen could not prevent obesity in HFD-fed mice either after 4 weeks or after 8 weeks. Consequently, the detected ameliorated glucose homeostasis in HFD-I must be related to something other than weight. The improvement of glucose tolerance could be due to a gain in lean mass or due to improved insulin sensitivity, which is reported in NLRP3 knockout mice.

Therefore, in addition to weighing the mice, their body composition was examined to see if the HFD led mainly to an increase in adipose tissue. Ebselen did not change the absolute and relative fat mass in the DIO mice. Regarding the relative amount of lean mass to body weight, both HFD groups had a lower percentage of lean mass compared to the lean groups, which is not surprising considering the increase in fat mass. There was no difference between the HFD groups. This is in accordance with the genetic knockout of the NLRP3 inflammasome, where

similar proportions of fat and lean body mass occur in HFD-fed WT and NLRP3 knockout mice [44].

Interestingly, Ebselen-treated HFD-fed mice showed an increase in lean mass compared with lean mice. The HFD alone did not increase lean mass. This change could explain the slightly improved glucose sensitivity of DIO mice treated with Ebselen [93].

Many studies state that blood plasma glucose levels and lean mass correlate negatively [94, 95]. However, in my opinion, it is highly questionable whether an increase of 5.85% of lean mass is sufficient to reverse glucose resistance at 1 month, and the increase of lean mass at the end of the experiment also does not explain the impeded glucose sensitivity after 2 months of HFD feeding. Unfortunately, we have no H-NMR data for 1 month of treatment.

We did not find any information about changes in lean mass in NLRP3 knockout mice in scientific literature. Furthermore, no mechanism of inflammasome activation leading to changes in lean mass was found. Hyperglycemia seems to lead to a higher vulnerability to skeletal muscle mass loss [96]. It could be interesting to analyze muscle tissue of mice further, in order to determine its NLRP3 levels. A possible explanation for the increase of lean mass is that on average, obese subjects have a higher amount of total lean mass, since the lean mass increases as a result of the higher demands on the muscles [97]. We do not have an explanation as to why the increase of absolute lean mass was limited to the HFD-I group and did not affect the HFD-C group.

Ebselen treatment did not change the absolute weight of the different fat compartments and did not change the normalized fat mass. HFD led to an obese phenotype, as expected. Even though we did not find any studies of absolute or relative mass of adipose tissue compartments in genetic NLRP3 knockout mice, we would have expected to find a reduction in visceral adipose tissue in mice treated with a pharmaceutical NLRP3 inhibitor, since genetic knockout leads to a smaller number of adipocytes in VAT but not in SAT.

The measurements with the metabolic cages revealed equal locomotion between the groups. As expected, the increased lean mass in HFD-I mice is not, therefore, due to increased locomotor activity in mice treated with Ebselen. A high-fat diet and subsequent obesity have been shown to decrease energy expenditure [98]. Therefore, it would be desirable to increase energy expenditure as a means of decreasing body weight. The metabolic cage measurements suggest a normal day/night rhythm in mice treated with Ebselen. The energy expenditure of lean mice

decreased compared to that of the lean control group. We could not find any literature stating that Ebselen decreases energy expenditure. Another study states the contrary effect - there the genetic ablation of components of the NLRP3 inflammasome led to a higher nighttime energy expenditure [44].

Unfortunately, 10 weeks on a high-fat diet was not sufficient to increase IL-1β levels in WAT and blood serum. We were, therefore, unable to look for any reduction in IL-β levels in the HFD-I groups. Elevated IL-1β levels in obesity are usually measured after 6 months on an HFD [43]. In most cases gene expression is measured [99]. The next step could be to look at the gene expression of different NLRP3 components.

In summary, Ebselen caused peritonitis in mice when injected intraperitoneally. Oral administration of Ebselen through drinking water had a slight effect on glucose metabolism after 1 month of treatment. We were not able to show any reduction of IL-1β levels in our mice. Ebselen is described as having multifunctional pharmaceutical effects, which means that these other non-NLRP3 inflammasome inhibiting effects could have an additional impact. The drug is known to act as a mimic of glutathione peroxidase [100]. The role of reactive oxygen species in the development of insulin resistance is not fully understood and there are conflicting theories stating that ROS improve [101] or worsen [102] insulin sensitivity. Therefore, we cannot exclude the possibility that Ebselen influenced the results through these mechanisms of action. On the other hand, reactive oxygen species (ROS) production seems to be crucial for NLRP3 inflammasome activation [103]. Thus, the anti-oxidative behavior of Ebselen could improve insulin sensitivity and avert complications in diabetes by reducing different ROS species. Additionally, Ebselen inhibits the DNA-binding capacity of different transcription factors, such as NF-κB [104]. This has been known for a long time, since it has not only been shown that obesity activates the IKKβ/NF-κB pathway, but also that obesity-induced insulin resistance is improved by pharmaceutical inhibitors of this pathway [105]. In T2D patients, a high dose of aspirin improves glucose metabolism [106]. Similarly, this pathway could be the leading mechanism of action. As described previously, NF-κb is also pivotal in the priming step of NLRP3 activation. As explained at the beginning of the discussion, an *in vitro* experiment where Ebselen is only added during the administration of LPS would be interesting in order to analyze the effect of Ebselen on the priming step and therefore on the NF-κb activation.

6.3 Conclusion and Outlook

Ebselen inhibited the release of IL-β in activated peritoneal macrophages and microglia. Yet we did not prove that Ebselen is an NLRP3 inflammasome inhibitor. To prove this, the concentration of other components of the NLRP3 inflammasome needed to be measured along with pyroptosis, an inflammatory form of programmed cell death which is dependent on caspase-1 activation. Based on these results, together with unpublished data from Veit Hornung suggesting that Ebselen is a potent inflammasome inhibitor, we continued with further *in vivo* experiments.

In the *in vivo* experiments, we showed that intraperitoneal injection of Ebselen leads to improved glucose resistance and a decrease in bodyweight. Importantly, this change could be attributed to the decrease of IL1β secretion or to the toxic effect of Ebselen, leading to death in the mice. Unfortunately, application of Ebselen through drinking water only yielded modest and temporary effects on glucose metabolism but did not affect weight gain in our HFD-IO mouse model. We cannot be sure whether this unsatisfactory effect is due to a lack of absorption and biological availability of the drug or the result of a minimal pharmacological effect of the inhibition of the NLRP3 inflammasome. It would have been interesting to measure the concentration of Ebselen in the periphery as well as in the CNS. This could be done by measuring selenium in the plasma of treated mice with liquid chromatography-mass spectrometry [107]. Different doses of Ebselen could also be used, since other *in vivo* data shows the absence of a clear dose response to Ebselen treatment [108].

Nevertheless, the formulation of this drug must be improved, as i.p. application induces toxic side effects and oral dosage in drinking water is insufficient to produce a pharmacological effect. If this could be achieved, it would be interesting to apply an NLRP3 inhibitor after the onset of obesity, since this would be a more realistic setup for the treatment of patients. Additionally, it would be necessary to analyze other organs which are affected by NLRP3 inflammasome activation in the context of diet-induced obesity.

Efficacious NLRP3 inhibitors are at an early stage of development. At the beginning of this thesis, no specific NLRP3 inhibitor was known, therefore the results of our experiments were awaited with great interest. Recently, two groups presented alternative NLRP3 inhibitors which proved to be bioavailable in the periphery and the CNS and show no sign of causing side effects. Studies using these drugs in the context of HFD-IO will evaluate the therapeutic potential for

impeding the development of obesity and insulin resistance as well as associated diseases [45, 109]. It has been shown that treatment with MCC950 could ameliorate insulin sensitivity in leptin receptor-deficient db/db mice and reduce the expression levels of inflammasome components, including NLRP3, ASC and caspase-1, and IL-1β in the hippocampus [110]. It would be interesting to use this detergent in the HFD-IO mouse model to explore whether a weight reduction could also be achieved. Additionally, it needs to be evaluated if a blockade of the NLRP3 inflammasome results in an increase of infection or sepsis.

Small molecules, such as Ebselen, MCC950 or BAY 11-7082, would be preferable to the use of biological drugs for targeting IL-1β or its receptor. These agents would probably cause less immunosuppression, be easier to apply and cost less. Therefore, more preclinical and clinical studies with specific NLRP3 inhibitors are needed to ensure the safety of this new approach.

7 References

1. Hennig, P., M. Garstkiewicz, S. Grossi, M. Di Filippo, L.E. French, and H.D. Beer, *The Crosstalk between Nrf2 and Inflammasomes.* Int J Mol Sci, 2018. **19**(2).

2. Haslam, D., N. Sattar, and M. Lean, *Obesity-time to wake up.* British Medical Journal, 2006. **7569**: p. 640.

3. Wardle, J., S. Carnell, C.M. Haworth, and R. Plomin, *Evidence for a strong genetic influence on childhood adiposity despite the force of the obesogenic environment.* The American journal of clinical nutrition, 2008. **87**(2): p. 398-404.

4. Turula, M., J. Kaprio, A. Rissanen, and M. Koskenvuo, *Body weight in the Finnish twin cohort.* Diabetes research and clinical practice, 1990. **10**: p. S33-S36.

5. Hill, J.O. and J.C. Peters, *Environmental contributions to the obesity epidemic.* Science, 1998. **280**(5368): p. 1371-1374.

6. Saris, W., A. Astrup, A. Prentice, H. Zunft, X. Formiguera, W. Verboeket-van de Venne, A. Raben, S. Poppitt, B. Seppelt, and S. Johnston, *Randomized controlled trial of changes in dietary carbohydrate/fat ratio and simple vs complex carbohydrates on body weight and blood lipids: the CARMEN study.* International journal of obesity, 2000. **24**(10): p. 1310-1318.

7. Hunter, G.R., T. Kekes-Szabo, S.W. Snyder, C. Nicholson, I. Nyikos, and L. Berland, *Fat distribution, physical activity, and cardiovascular risk factors.* Medicine and science in sports and exercise, 1997. **29**(3): p. 362-369.

8. Organization, W.H., *Physical inactivity: a global public health problem. 2010.* URL www. who. int/dietphysicalactivity/factsheet_inactivity/en/. Part I APPENDIX, 2012.

9. Nguyen, D.M. and H.B. El-Serag, *The epidemiology of obesity.* Gastroenterology Clinics of North America, 2010. **39**(1): p. 1-7.

10. Group, L.A.R., *Long term effects of a lifestyle intervention on weight and cardiovascular risk factors in individuals with type 2 diabetes: four year results of the Look AHEAD trial.* Archives of internal medicine, 2010. **170**(17): p. 1566.

11. Knowler, W.C., S.E. Fowler, R.F. Hamman, C.A. Christophi, H.J. Hoffman, A.T. Brenneman, J.O. Brown-Friday, R. Goldberg, E. Venditti, and D.M. Nathan, *10-year follow-up of diabetes incidence and weight loss in the Diabetes Prevention Program Outcomes Study.* Lancet (London, England), 2009. **374**(9702): p. 1677-1686.

12. Wing, R.R. and S. Phelan, *Long-term weight loss maintenance.* Am J Clin Nutr, 2005. **82**(1 Suppl): p. 222S-225S.

13. Organization, W.H., *Global status report on noncommunicable diseases 2014*. 2014, World Health Organization.

14. Look, A.R.G., E.W. Gregg, J.M. Jakicic, G. Blackburn, P. Bloomquist, G.A. Bray, J.M. Clark, M. Coday, J.M. Curtis, C. Egan, M. Evans, J. Foreyt, G. Foster, H.P. Hazuda, J.O. Hill, E.S. Horton, V.S. Hubbard, R.W. Jeffery, K.C. Johnson, A.E. Kitabchi, W.C.

Knowler, A. Kriska, W. Lang, C.E. Lewis, M.G. Montez, D.M. Nathan, R.H. Neiberg, J. Patricio, A. Peters, X. Pi-Sunyer, H. Pownall, B. Redmon, J. Regensteiner, J. Rejeski, P.M. Ribisl, M. Safford, K. Stewart, D. Trence, T.A. Wadden, R.R. Wing, and S.Z. Yanovski, *Association of the magnitude of weight loss and changes in physical fitness with long-term cardiovascular disease outcomes in overweight or obese people with type 2 diabetes: a post-hoc analysis of the Look AHEAD randomised clinical trial.* Lancet Diabetes Endocrinol, 2016. **4**(11): p. 913-921.

15. Hotamisligil, G.S., P. Peraldi, A. Budavari, R. Ellis, M.F. White, and B.M. Spiegelman, *IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance.* Science, 1996. **271**(5249): p. 665-8.

16. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.

17. Lumeng, C.N., J. Liu, L. Geletka, C. Delaney, J. Delproposto, A. Desai, K. Oatmen, G. Martinez-Santibanez, A. Julius, S. Garg, and R.L. Yung, *Aging is associated with an increase in T cells and inflammatory macrophages in visceral adipose tissue.* J Immunol, 2011. **187**(12): p. 6208-16.

18. de Heredia, F.P., S. Gomez-Martinez, and A. Marcos, *Obesity, inflammation and the immune system.* Proc Nutr Soc, 2012. **71**(2): p. 332-8.

19. Ferrante, A.W., Jr., *Obesity-induced inflammation: a metabolic dialogue in the language of inflammation.* J Intern Med, 2007. **262**(4): p. 408-14.

20. Ralston, J.C., C.L. Lyons, E.B. Kennedy, A.M. Kirwan, and H.M. Roche, *Fatty Acids and NLRP3 Inflammasome-Mediated Inflammation in Metabolic Tissues.* Annu Rev Nutr, 2017. **37**: p. 77-102.

21. Gregor, M.F. and G.S. Hotamisligil, *Inflammatory mechanisms in obesity.* Annu Rev Immunol, 2011. **29**: p. 415-45.

22. van Herpen, N.A. and V.B. Schrauwen-Hinderling, *Lipid accumulation in non-adipose tissue and lipotoxicity.* Physiol Behav, 2008. **94**(2): p. 231-41.

23. Krahmer, N., R.V. Farese, Jr., and T.C. Walther, *Balancing the fat: lipid droplets and human disease.* EMBO Mol Med, 2013. **5**(7): p. 973-83.

24. Xu, H., G.T. Barnes, Q. Yang, G. Tan, D. Yang, C.J. Chou, J. Sole, A. Nichols, J.S. Ross, L.A. Tartaglia, and H. Chen, *Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance.* J Clin Invest, 2003. **112**(12): p. 1821-30.

25. Weisberg, S.P., D. McCann, M. Desai, M. Rosenbaum, R.L. Leibel, and A.W. Ferrante, Jr., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.

26. Thaler, J.P. and M.W. Schwartz, *Minireview: Inflammation and obesity pathogenesis: the hypothalamus heats up.* Endocrinology, 2010. **151**(9): p. 4109-15.

27. Milanski, M., G. Degasperi, A. Coope, J. Morari, R. Denis, D.E. Cintra, D.M. Tsukumo, G. Anhe, M.E. Amaral, H.K. Takahashi, R. Curi, H.C. Oliveira, J.B. Carvalheira, S. Bordin, M.J. Saad, and L.A. Velloso, *Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity.* J Neurosci, 2009. **29**(2): p. 359-70.

28. Obici, S. and L. Rossetti, *Minireview: nutrient sensing and the regulation of insulin action and energy balance.* Endocrinology, 2003. **144**(12): p. 5172-8.

29. Martinon, F., A. Mayor, and J. Tschopp, *The inflammasomes: guardians of the body.* Annu Rev Immunol, 2009. **27**: p. 229-65.

30. Reynolds, C.M., F.C. McGillicuddy, K.A. Harford, O.M. Finucane, K.H. Mills, and H.M. Roche, *Dietary saturated fatty acids prime the NLRP3 inflammasome via TLR4 in dendritic cells-implications for diet-induced insulin resistance.* Mol Nutr Food Res, 2012. **56**(8): p. 1212-22.

31. De Nardo, D. and E. Latz, *NLRP3 inflammasomes link inflammation and metabolic disease.* Trends Immunol, 2011. **32**(8): p. 373-9.

32. Chen, G.Y. and G. Nunez, *Sterile inflammation: sensing and reacting to damage.* Nat Rev Immunol, 2010. **10**(12): p. 826-37.

33. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta.* Mol Cell, 2002. **10**(2): p. 417-26.

34. Sutterwala, F.S., Y. Ogura, M. Szczepanik, M. Lara-Tejero, G.S. Lichtenberger, E.P. Grant, J. Bertin, A.J. Coyle, J.E. Galán, and P.W. Askenase, *Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1.* Immunity, 2006. **24**(3): p. 317-327.

35. Ghayur, T., S. Banerjee, M. Hugunin, D. Butler, L. Herzog, A. Carter, L. Quintal, L. Sekut, R. Talanian, M. Paskind, W. Wong, R. Kamen, D. Tracey, and H. Allen, *Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production.* Nature, 1997. **386**(6625): p. 619-23.

36. Shi, J., Y. Zhao, K. Wang, X. Shi, Y. Wang, H. Huang, Y. Zhuang, T. Cai, F. Wang, and F. Shao, *Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death.* Nature, 2015. **526**(7575): p. 660-5.

37. Bauernfeind, F.G., G. Horvath, A. Stutz, E.S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B.G. Monks, K.A. Fitzgerald, V. Hornung, and E. Latz, *Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression.* J Immunol, 2009. **183**(2): p. 787-91.

38. Schroder, K. and J. Tschopp, *The inflammasomes.* Cell, 2010. **140**(6): p. 821-32.

39. Menu, P. and J.E. Vince, *The NLRP3 inflammasome in health and disease: the good, the bad and the ugly.* Clin Exp Immunol, 2011. **166**(1): p. 1-15.

40. Yin, Z., T. Deng, L.E. Peterson, R. Yu, J. Lin, D.J. Hamilton, P.R. Reardon, V. Sherman, G.E. Winnier, M. Zhan, C.J. Lyon, S.T. Wong, and W.A. Hsueh, *Transcriptome analysis of human adipocytes implicates the NOD-like receptor pathway in obesity-induced adipose inflammation.* Mol Cell Endocrinol, 2014. **394**(1-2): p. 80-7.

41. Stienstra, R., L.A. Joosten, T. Koenen, B. van Tits, J.A. van Diepen, S.A. van den Berg, P.C. Rensen, P.J. Voshol, G. Fantuzzi, and A. Hijmans, *The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity.* Cell metabolism, 2010. **12**(6): p. 593-605.

42. Esser, N., L. L'homme, A. De Roover, L. Kohnen, A.J. Scheen, M. Moutschen, J. Piette, S. Legrand-Poels, and N. Paquot, *Obesity phenotype is related to NLRP3 inflammasome activity and immunological profile of visceral adipose tissue.* Diabetologia, 2013. **56**(11): p. 2487-2497.

43. Vandanmagsar, B., Y.H. Youm, A. Ravussin, J.E. Galgani, K. Stadler, R.L. Mynatt, E. Ravussin, J.M. Stephens, and V.D. Dixit, *The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance.* Nat Med, 2011. **17**(2): p. 179-88.

44. Stienstra, R., J.A. van Diepen, C.J. Tack, M.H. Zaki, F.L. van de Veerdonk, D. Perera, G.A. Neale, G.J. Hooiveld, A. Hijmans, I. Vroegrijk, S. van den Berg, J. Romijn, P.C. Rensen, L.A. Joosten, M.G. Netea, and T.D. Kanneganti, *Inflammasome is a central player in the induction of obesity and insulin resistance.* Proc Natl Acad Sci U S A, 2011. **108**(37): p. 15324-9.

45. Youm, Y.H., A. Adijiang, B. Vandanmagsar, D. Burk, A. Ravussin, and V.D. Dixit, *Elimination of the NLRP3-ASC inflammasome protects against chronic obesity-induced pancreatic damage.* Endocrinology, 2011. **152**(11): p. 4039-45.

46. Major, C.D. and B.A. Wolf, *Interleukin-1beta stimulation of c-Jun NH(2)-terminal kinase activity in insulin-secreting cells: evidence for cytoplasmic restriction.* Diabetes, 2001. **50**(12): p. 2721-8.

47. Spranger, J., A. Kroke, M. Möhlig, K. Hoffmann, M.M. Bergmann, M. Ristow, H. Boeing, and A.F. Pfeiffer, *Inflammatory cytokines and the risk to develop type 2 diabetes results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study.* Diabetes, 2003. **52**(3): p. 812-817.

48. Everett, B.M., M.Y. Donath, A.D. Pradhan, T. Thuren, P. Pais, J.C. Nicolau, R.J. Glynn, P. Libby, and P.M. Ridker, *Anti-Inflammatory Therapy With Canakinumab for the Prevention and Management of Diabetes.* J Am Coll Cardiol, 2018. **71**(21): p. 2392-2401.

49. Sun, S., S. Xia, Y. Ji, S. Kersten, and L. Qi, *The ATP-P2X7 signaling axis is dispensable for obesity-associated inflammasome activation in adipose tissue.* Diabetes, 2012. **61**(6): p. 1471-1478.

50. Rabinowitz, S.S. and S. Gordon, *Macrosialin, a macrophage-restricted membrane sialoprotein differentially glycosylated in response to inflammatory stimuli.* J Exp Med, 1991. **174**(4): p. 827-36.

51. Burguillos, M.A., T. Deierborg, E. Kavanagh, A. Persson, N. Hajji, A. Garcia-Quintanilla, J. Cano, P. Brundin, E. Englund, J.L. Venero, and B. Joseph, *Caspase signalling controls microglia activation and neurotoxicity.* Nature, 2011. **472**(7343): p. 319-24.

52. Sanz, J.M. and F. Di Virgilio, *Kinetics and mechanism of ATP-dependent IL-1 beta release from microglial cells.* J Immunol, 2000. **164**(9): p. 4893-8.

53. Ferrari, D., P. Chiozzi, S. Falzoni, S. Hanau, and F. Di Virgilio, *Purinergic modulation of interleukin-1 beta release from microglial cells stimulated with bacterial endotoxin.* J Exp Med, 1997. **185**(3): p. 579-82.

54. Cantó, C., R.H. Houtkooper, E. Pirinen, D.Y. Youn, M.H. Oosterveer, Y. Cen, P.J. Fernandez-Marcos, H. Yamamoto, P.A. Andreux, and P. Cettour-Rose, *The NAD+ precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity.* Cell metabolism, 2012. **15**(6): p. 838-847.

55. Koonen, D.P., R.L. Jacobs, M. Febbraio, M.E. Young, C.-L.M. Soltys, H. Ong, D.E. Vance, and J.R. Dyck, *Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity.* Diabetes, 2007. **56**(12): p. 2863-2871.

56. Andrikopoulos, S., A.R. Blair, N. Deluca, B.C. Fam, and J. Proietto, *Evaluating the glucose tolerance test in mice.* American Journal of Physiology-Endocrinology and Metabolism, 2008. **295**(6): p. E1323-E1332.

57. Pitha, J., J. Milecki, H. Fales, L. Pannell, and K. Uekama, *Hydroxypropyl-β-cyclodextrin: preparation and characterization; effects on solubility of drugs.* International journal of Pharmaceutics, 1986. **29**(1): p. 73-82.

58. Brodsky, S.V., O. Gealekman, J. Chen, F. Zhang, N. Togashi, M. Crabtree, S.S. Gross, A. Nasjletti, and M.S. Goligorsky, *Prevention and reversal of premature endothelial cell senescence and vasculopathy in obesity-induced diabetes by ebselen.* Circulation research, 2004. **94**(3): p. 377-384.

59. Chander, P.N., O. Gealekman, S.V. Brodsky, S. Elitok, A. Tojo, M. Crabtree, S.S. Gross, and M.S. Goligorsky, *Nephropathy in Zucker diabetic fat rat is associated with oxidative and nitrosative stress: prevention by chronic therapy with a peroxynitrite scavenger ebselen.* Journal of the American Society of Nephrology, 2004. **15**(9): p. 2391-2403.

60. Singh, N., A.C. Halliday, J.M. Thomas, O.V. Kuznetsova, R. Baldwin, E.C. Woon, P.K. Aley, I. Antoniadou, T. Sharp, S.R. Vasudevan, and G.C. Churchill, *A safe lithium mimetic for bipolar disorder.* Nat Commun, 2013. **4**: p. 1332.

61. Galvao, J., B. Davis, M. Tilley, E. Normando, M.R. Duchen, and M.F. Cordeiro, *Unexpected low-dose toxicity of the universal solvent DMSO.* FASEB J, 2014. **28**(3): p. 1317-30.

62. Gould, S. and R.C. Scott, *2-Hydroxypropyl-beta-cyclodextrin (HP-beta-CD): a toxicology review.* Food Chem Toxicol, 2005. **43**(10): p. 1451-9.

63. Pitha, J. and J. Pitha, *Amorphous water-soluble derivatives of cyclodextrins: nontoxic dissolution enhancing excipients.* J Pharm Sci, 1985. **74**(9): p. 987-90.

64. Tan, S.M., A. Sharma, N. Stefanovic, and J.B. de Haan, *Late-intervention study with ebselen in an experimental model of type 1 diabetic nephropathy.* Free radical research, 2015. **49**(3): p. 219-227.

65. Hanavan, P.D., C.R. Borges, B.A. Katchman, D.O. Faigel, T.H. Ho, C.-T. Ma, E.A. Sergienko, N. Meurice, J.L. Petit, and D.F. Lake, *Ebselen inhibits QSOX1 enzymatic activity and suppresses invasion of pancreatic and renal cancer cell lines.* Oncotarget, 2015. **6**(21): p. 18418.

66. Tan, S.M., A. Sharma, D.Y. Yuen, N. Stefanovic, G. Krippner, G. Mugesh, Z. Chai, and J.B. de Haan, *The modified selenenyl amide, M-hydroxy ebselen, attenuates diabetic nephropathy and diabetes-associated atherosclerosis in ApoE/GPx1 double knockout mice.* 2013.

67. Yamaguchi, T., K. Sano, K. Takakura, I. Saito, Y. Shinohara, T. Asano, and H. Yasuhara, *Ebselen in acute ischemic stroke a placebo-controlled, double-blind clinical trial.* Stroke, 1998. **29**(1): p. 12-17.

68. Brydges, S.D., L. Broderick, M.D. McGeough, C.A. Pena, J.L. Mueller, and H.M. Hoffman, *Divergence of IL-1, IL-18, and cell death in NLRP3 inflammasomopathies.* J Clin Invest, 2013. **123**(11): p. 4695-705.

69. Vague, J., *The degree of masculine differentiation of obesities a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease.* The American journal of clinical nutrition, 1956. **4**(1): p. 20-34.

70. Montague, C.T. and S. O'Rahilly, *The perils of portliness: causes and consequences of visceral adiposity.* Diabetes, 2000. **49**(6): p. 883-888.

71. Zoico, E., V. Di Francesco, G. Mazzali, R. Vettor, F. Fantin, L. Bissoli, S. Guariento, O. Bosello, and M. Zamboni, *Adipocytokines, fat distribution, and insulin resistance in elderly men and women.* J Gerontol A Biol Sci Med Sci, 2004. **59**(9): p. M935-9.

72. Jin, C. and R.A. Flavell, *Molecular mechanism of NLRP3 inflammasome activation.* J Clin Immunol, 2010. **30**(5): p. 628-31.

73. Li, L.H., T.C. Ju, C.Y. Hsieh, W.C. Dong, W.T. Chen, K.F. Hua, and W.J. Chen, *A synthetic cationic antimicrobial peptide inhibits inflammatory response and the NLRP3 inflammasome by neutralizing LPS and ATP.* PLoS One, 2017. **12**(7): p. e0182057.

74. Xie, Q., W.W. Shen, J. Zhong, C. Huang, L. Zhang, and J. Li, *Lipopolysaccharide/adenosine triphosphate induces IL1beta and IL-18 secretion through the NLRP3 inflammasome in RAW264.7 murine macrophage cells.* Int J Mol Med, 2014. **34**(1): p. 341-9.

75. Drost, N., *Characterization and manipulation of myeloid cells in the healthy and diseased Central Nervous System*, U. Charité, Editor. 2015: unpublished.

76. Jabaut, J., J.L. Ather, A. Taracanova, M.E. Poynter, and K. Ckless, *Mitochondria-targeted drugs enhance Nlrp3 inflammasome-dependent IL-1beta secretion in association with alterations in cellular redox and energy status.* Free Radic Biol Med, 2013. **60**: p. 233-45.

77. Buckman, L.B., A.H. Hasty, D.K. Flaherty, C.T. Buckman, M.M. Thompson, B.K. Matlock, K. Weller, and K.L. Ellacott, *Obesity induced by a high-fat diet is associated with increased immune cell entry into the central nervous system.* Brain Behav Immun, 2014. **35**: p. 33-42.

78. Erion, J.R., M. Wosiski-Kuhn, A. Dey, S. Hao, C.L. Davis, N.K. Pollock, and A.M. Stranahan, *Obesity elicits interleukin 1-mediated deficits in hippocampal synaptic plasticity.* J Neurosci, 2014. **34**(7): p. 2618-31.

79. Spencer, S.J., H. D'Angelo, A. Soch, L.R. Watkins, S.F. Maier, and R.M. Barrientos, *High-fat diet and aging interact to produce neuroinflammation and impair hippocampal- and amygdalar-dependent memory.* Neurobiol Aging, 2017. **58**: p. 88-101.

80. Owyang, A.M., H. Issafras, J. Corbin, K. Ahluwalia, P. Larsen, E. Pongo, M. Handa, A.H. Horwitz, M.K. Roell, M. Haak-Frendscho, and L. Masat, *XOMA 052, a potent, high-affinity monoclonal antibody for the treatment of IL-1beta-mediated diseases.* MAbs, 2011. **3**(1): p. 49-60.

81. Juliana, C., T. Fernandes-Alnemri, J. Wu, P. Datta, L. Solorzano, J.W. Yu, R. Meng, A.A. Quong, E. Latz, C.P. Scott, and E.S. Alnemri, *Anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome.* J Biol Chem, 2010. **285**(13): p. 9792-802.

82. Zorman, J., P. Susjan, and I. Hafner-Bratkovic, *Shikonin Suppresses NLRP3 and AIM2 Inflammasomes by Direct Inhibition of Caspase-1.* PLoS One, 2016. **11**(7): p. e0159826.

83. Shirasuna, K., H. Takano, K. Seno, A. Ohtsu, T. Karasawa, M. Takahashi, A. Ohkuchi, H. Suzuki, S. Matsubara, H. Iwata, and T. Kuwayama, *Palmitic acid induces interleukin-1beta secretion via NLRP3 inflammasomes and inflammatory responses through ROS production in human placental cells.* J Reprod Immunol, 2016. **116**: p. 104-12.

84. Turner, P.V., T. Brabb, C. Pekow, and M.A. Vasbinder, *Administration of substances to laboratory animals: routes of administration and factors to consider.* J Am Assoc Lab Anim Sci, 2011. **50**(5): p. 600-13.

85. Stienstra, R., C.J. Tack, T.D. Kanneganti, L.A. Joosten, and M.G. Netea, *The inflammasome puts obesity in the danger zone.* Cell Metab, 2012. **15**(1): p. 10-8.

86. Chew, P., D.Y. Yuen, P. Koh, N. Stefanovic, M.A. Febbraio, I. Kola, M.E. Cooper, and J.B. de Haan, *Site-specific antiatherogenic effect of the antioxidant ebselen in the diabetic apolipoprotein E-deficient mouse.* Arterioscler Thromb Vasc Biol, 2009. **29**(6): p. 823-30.

87. Tripathi, D.N. and G.B. Jena, *Ebselen attenuates cyclophosphamide-induced oxidative stress and DNA damage in mice.* Free Radic Res, 2008. **42**(11-12): p. 966-77.

88. Dhanarajan, R., P. Abraham, and B. Isaac, *Protective effect of ebselen, a selenoorganic drug, against gentamicin-induced renal damage in rats.* Basic Clin Pharmacol Toxicol, 2006. **99**(3): p. 267-72.

89. Calarco, C.A., S. Lee, and M.R. Picciotto, *Access to nicotine in drinking water reduces weight gain without changing caloric intake on high fat diet in male C57BL/6J mice.* Neuropharmacology, 2017. **123**: p. 210-220.

90. Wang, H., W. Capell, J.H. Yoon, S. Faubel, and R.H. Eckel, *Obesity development in caspase-1-deficient mice.* Int J Obes (Lond), 2014. **38**(1): p. 152-5.

91. Tremaroli, V. and F. Backhed, *Functional interactions between the gut microbiota and host metabolism.* Nature, 2012. **489**(7415): p. 242-9.

92. Netea, M.G., L.A. Joosten, E. Lewis, D.R. Jensen, P.J. Voshol, B.J. Kullberg, C.J. Tack, H. van Krieken, S.H. Kim, A.F. Stalenhoef, F.A. van de Loo, I. Verschueren, L. Pulawa, S. Akira, R.H. Eckel, C.A. Dinarello, W. van den Berg, and J.W. van der Meer, *Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance.* Nat Med, 2006. **12**(6): p. 650-6.

93. Nam, S.Y., K.R. Kim, B.S. Cha, Y.D. Song, S.K. Lim, H.C. Lee, and K.B. Huh, *Low-dose growth hormone treatment combined with diet restriction decreases insulin resistance by reducing visceral fat and increasing muscle mass in obese type 2 diabetic patients.* Int J Obes Relat Metab Disord, 2001. **25**(8): p. 1101-7.

94. Ridker, P.M., C.P. Howard, V. Walter, B. Everett, P. Libby, J. Hensen, T. Thuren, and C.P.I. Group, *Effects of interleukin-1beta inhibition with canakinumab on hemoglobin A1c, lipids, C-reactive protein, interleukin-6, and fibrinogen: a phase IIb randomized, placebo-controlled trial.* Circulation, 2012. **126**(23): p. 2739-48.

95. van Asseldonk, E.J., R. Stienstra, T.B. Koenen, L.A. Joosten, M.G. Netea, and C.J. Tack, *Treatment with Anakinra improves disposition index but not insulin sensitivity in nondiabetic subjects with the metabolic syndrome: a randomized, double-blind, placebo-controlled study.* J Clin Endocrinol Metab, 2011. **96**(7): p. 2119-26.

96. Kalyani, R.R., M. Corriere, and L. Ferrucci, *Age-related and disease-related muscle loss: the effect of diabetes, obesity, and other diseases.* Lancet Diabetes Endocrinol, 2014. **2**(10): p. 819-29.

97. Forbes, G.B. and S.L. Welle, *Lean body mass in obesity.* Int J Obes, 1983. **7**(2): p. 99-107.

98. Storlien, L.H., D.E. James, K.M. Burleigh, D.J. Chisholm, and E.W. Kraegen, *Fat feeding causes widespread in vivo insulin resistance, decreased energy expenditure, and obesity in rats.* Am J Physiol, 1986. **251**(5 Pt 1): p. E576-83.

99. Vieira, V.J., R.J. Valentine, K.R. Wilund, and J.A. Woods, *Effects of diet and exercise on metabolic disturbances in high-fat diet-fed mice.* Cytokine, 2009. **46**(3): p. 339-45.

100. Azad, G.K. and R.S. Tomar, *Ebselen, a promising antioxidant drug: mechanisms of action and targets of biological pathways.* Mol Biol Rep, 2014. **41**(8): p. 4865-79.

101. Gonzalez, F., N.S. Rote, J. Minium, and J.P. Kirwan, *Reactive oxygen species-induced oxidative stress in the development of insulin resistance and hyperandrogenism in polycystic ovary syndrome.* J Clin Endocrinol Metab, 2006. **91**(1): p. 336-40.

102. Houstis, N., E.D. Rosen, and E.S. Lander, *Reactive oxygen species have a causal role in multiple forms of insulin resistance.* Nature, 2006. **440**(7086): p. 944-8.

103. Tschopp, J. and K. Schroder, *NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production?* Nat Rev Immunol, 2010. **10**(3): p. 210-5.

104. Larabee, J.L., J.R. Hocker, and J.S. Hanas, *Mechanisms of inhibition of zinc-finger transcription factors by selenium compounds ebselen and selenite.* J Inorg Biochem, 2009. **103**(3): p. 419-26.

105. Yuan, M., N. Konstantopoulos, J. Lee, L. Hansen, Z.W. Li, M. Karin, and S.E. Shoelson, *Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta.* Science, 2001. **293**(5535): p. 1673-7.

106. Hundal, R.S., K.F. Petersen, A.B. Mayerson, P.S. Randhawa, S. Inzucchi, S.E. Shoelson, and G.I. Shulman, *Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes.* J Clin Invest, 2002. **109**(10): p. 1321-6.

107. Kil, J., E. Lobarinas, C. Spankovich, S.K. Griffiths, P.J. Antonelli, E.D. Lynch, and C.G. Le Prell, *Safety and efficacy of ebselen for the prevention of noise-induced hearing loss: a randomised, double-blind, placebo-controlled, phase 2 trial.* Lancet, 2017. **390**(10098): p. 969-979.

108. Pourbakht, A. and T. Yamasoba, *Ebselen attenuates cochlear damage caused by acoustic trauma.* Hear Res, 2003. **181**(1-2): p. 100-8.

109. Coll, R.C., A.A. Robertson, J.J. Chae, S.C. Higgins, R. Munoz-Planillo, M.C. Inserra, I. Vetter, L.S. Dungan, B.G. Monks, A. Stutz, D.E. Croker, M.S. Butler, M. Haneklaus, C.E. Sutton, G. Nunez, E. Latz, D.L. Kastner, K.H. Mills, S.L. Masters, K. Schroder, M.A. Cooper, and L.A. O'Neill, *A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases.* Nat Med, 2015. **21**(3): p. 248-55.

110. Zhai, Y., X. Meng, T. Ye, W. Xie, G. Sun, and X. Sun, *Inhibiting the NLRP3 Inflammasome Activation with MCC950 Ameliorates Diabetic Encephalopathy in db/db Mice.* Molecules, 2018. **23**(3).

8 Eidesstattliche Versicherung

Ich, Franziska Antonia Elisabeth Metke, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Impact of inflammasome inhibition on the development of insulin resistance in the context of diet induced obesity“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Erstbetreuer, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Datum Unterschrift

9 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

10 Acknowledgements

At this point I would like to thank a group of people, without whom the realization of this thesis would not have been possible.

First of all, I want to thank Prof. Heppner for giving me the opportunity to pursue my PhD in his institute. Thank you for your guidance and support.

A very special and sincere thank you to Kelly Miller and Prof. Frank Heppner for being a great supervisor.

I want to thank all the members of the Department of Neuropathology; including the lab technicians, doctorates and postdocs for their help and assistance whenever problems or questions came up.

I also thank the colleagues of the Department of Endocrinology, Diabetes and Nutrition and the Center of Cardiovascular Research who made it possible for me to use the metabolic cages and the body composition NMR.

Last, but not least, I want to thank my family for their unconditional support.