Factors impacting the hepatic selenoprotein expression in matters of critical illness

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Abbreviation

AG	Aminoglycoside	**IL-1β**	Interleukin-1β
AP-1	Activator protein 1	**IL-6**	Interleukin-6
ApoER2	Apolipoprotein E receptor 2	**kb**	kilo base
APS	Ammonium persulfate	**KCL**	Potassium chloride
ATP	Adenosine triphosphate	**kDa**	kilo Dalton
BCA	Bicinchoninic acid	**LB**	Luria-Bertani broth
Bp	Base pair(s)	**LPS**	Lipopolysaccharide
BSA	Bovine serum albumin	**Lrp2**	Low density lipoprotein-related protein 2
C	Celsius	**M**	Molar (moles/litre)
cDNA	Complementary DNA	**MAPK**	Mitogen activated protein kinase
ChiPSeq	Chromatin Immunoprecipitation sequencing	**mRNA**	Messenger RNA
CRP	C-reactive protein	**NaCl**	Sodium chloride
CP	Ceruloplasmin	**NADPH**	Nicotinamide adenine dinucleotide phosphate
Cys	Cysteine	**NCBI**	National Center for Biotechnology Information
DIO	Iodothyronine deiodinase	**NF-κB**	Nuclear factor kappa-light-chain-enhancer of activated B cells
DNA	Deoxyribonucleic acid	**NMD**	Nonsense-mediated mRNA decay
DTT	Dithiothreitol	**PBS**	Phosphate buffered saline
E. coli	Escherichia coli	**PCR**	polymerase chain reaction
EDTA	Ethylenediaminetetraacetic acid	**PSTK**	O-phosphoseryl-tRNA(Sec) kinase
EFsec	Selenocysteine-specific elongation factor	**PTU**	propylthiouracil
ELISA	Enzyme-linked Immunosorbent Assay	**qRT-PCR**	quantitative RealTime-PCR
ER	Endoplasmic reticulum	**RE**	Response element
FBS	Fetal calf serum	**RLuc**	Renilla Luciferase
FLuc	Firefly Luciferase	**RNA**	Ribonucleic acid
GEN	gentamicin	**RLU**	Relative light unit
GPX	Glutathione peroxidase	**ROS**	Reactive oxygen species
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	**RPM**	Revolutions per minute
HRP	Horseradish peroxidase	**RT**	Room temperature
HPRT	Hypoxanthine-guanine phosphoribosyltransferase	**rT_3**	Reverse T3
ICU	Intensive care unit	**RU**	Relative units

SAP	Shrimp alkaline phosphatase	**STAT3**	Signal transducer and activator of transcription 3
SBP2	Selenocysteine insertion sequence-binding protein 2	**SV40**	Simian vacuolating virus 40
SDS	Sodium dodecyl sulphate	**T_2**	3,5-Diiodo-L-thyronine
SEAP	Secreted embryonic alkaline phosphatase	**T_3**	Triiodothyronine
Sec	Selenocysteine	**T_4**	Thyroxine
SECIS	Selenocysteine inserstion sequence	**TAE**	Tris-acetate-EDTA
SecS	Selenocysteinyl-tRNA(Sec) synthase	**TBS**	Tris-buffered saline
SelenBP1	Selenium-binding protein 1	**TEMED**	Tetramethylethylenediamine
SelK	Selenoprotein K	**TH**	Thyroid hormone
SelN	Selenoprotein N	**TGFβ**	Transforming growth factor beta
SelS	Selenoprotein S		
SEM	Standard error of the mean	**TNFα**	Tumour necrosis factor alpha
SeMet	Selenomethionine	**TRIS**	Tris(hydroxymethyl)aminomethane
SEPP	Selenoprotein P	**tRNA**	Transfer RNA
SEPX	Selenoprotein X	**TXN**	Thioredoxin
SeRS	Seryl-tRNA(Ser/Sec) synthetase	**TXNRD**	Thioredoxin reductase
SNP	Single nucleotide polymorphism	**TXRF**	Total reflection x-ray fluorescence
SOD	Superoxide dismutase	**UTR**	Untranslated region
SPS2	Selenophosphate synthetase 2		

The **nomenclature** of genes and proteins largely corresponds to the current official guidelines. Genes and transcripts are written in italics. Human genes and proteins are represented in uppercase letters, whereas murine genes and proteins are represented in first letter uppercase followed by lowercase letters.

Examples:	(gene, human) *SEPP*	(protein, human) SEPP
	(gene, mouse) *Sepp*	(protein, mouse) Sepp
	(gene, human) *DIO1*	(protein, human) DIO1
	(gene, mouse) *Dio1*	(protein, mouse) Dio1

Summary

Selenium (Se) is an essential trace element as part of the 21st proteinogenic amino acid selenocysteine (Sec) in selenoproteins. Selenoproteins play important roles in redox-regulating signal pathways, the antioxidant defence, thyroid hormone metabolism and immunoregulation. Se-metabolism is controlled by hepatocytes synthesizing and secreting the Se-transporter selenoprotein P (SEPP). Circulating SEPP declines in critical illness, e.g. sepsis causing low serum Se-levels, which in turn negatively correlates with mortality. Sepsis triggers excessive production of pro-inflammatory cytokines including interleukin-6 (IL-6), causing oxidative stress, tissue damage and organ dysfunction. Aminoglycoside (AG) antibiotics are often applied in severe sepsis in order to fight infection. AG induce mRNA misinterpretation including the stop codon UGA. The recoding of UGA and the presence of a selenoprotein-specific Sec-insertion sequence (SECIS) element within the mRNA are essentially required during selenoproteins biosynthesis.

As liver is the major organ regulating Se-metabolism, the molecular interplay between pro-inflammatory cytokines (i.e. Interleukin-6 (IL-6), Interleukin-1β, and tumour necrosis factor α), aminoglycoside antibiotics (i.e. G418 and gentamicin) and Se-status on selenoprotein expression was investigated in hepatocytes.

IL-6 strongly reduced the level of SEPP mRNA and secreted SEPP in a dose-dependent manner. Likewise, expression of selenoenzyme iodothyronine deiodinase type 1 (DIO1) declined at the transcript, protein and enzyme activity level. The effects of IL-6 on the expression of antioxidative acting glutathione peroxidases (GPX) were isozyme-specific; while transcript level of *GPX2* increased and those of *GPX4* decreased, *GPX1* remained unaffected. These IL-6-dependent effects were reflected in reporter gene experiments of *SEPP*, *DIO1*, *GPX2*, and *GPX4* promoter constructs and point to direct transcriptional effects of IL-6. A combination of IL-6, Interleukin-1β and tumour necrosis factor α resulted in more prominent decrease in SEPP and DIO1 expression, while the induction of GPX enzyme activity was greater in comparison to IL-6 alone. These results highlight a redistribution of selenoprotein expression in favour of certain selenoproteins of high importance in inflammatory diseases.

In an attempt to better characterise the effects of AG on selenoprotein translation, the SECIS-elements of *GPX1*, *GPX4* and *SEPP* transcripts were cloned into a reporter system and analysed for their response to AG and Se. The results indicate that the correct co-translational Sec-insertion depends on the Se-status, AG concentration and the specific SECIS-element. At both transcriptional and translational levels, SEPP levels were strongly increased in response to AG, whereas the expression and enzyme activity of GPX1, GPX2, GPX4 and

DIO1 were affected to a lower degree. Analysis with total reflection X-ray fluorescence indicate that the Se-content of SEPP was significantly reduced by AG and depends on Se-status. Especially the importance of Se-status to overcome the disrupting and suppressing effects of AG and pro-inflammatory cytokines is of high clinical relevance. It directly highlights Se-deficiency as a central risk factor for negative side effects and suggests Se-supplementation as a likely meaningful intervention strategy during critical illness.

Zusammenfassung

Selen ist ein essentielles Spurenelement, welches seine Funktion in Form der 21. proteinogenen Aminosäure Selenocystein (Sec) in Selenoproteinen entfaltet. Selenoproteine spielen eine wichtige Rolle in Redox-regulierenden Signalwegen, in der antioxidativen Abwehr, im Schilddrüsenhormon-Stoffwechsel und bei Immunreaktionen. Der Selenmetabolismus wird von Hepatozyten gesteuert, welche das Selen-Transportprotein Selenoprotein P (SEPP) synthetisieren und sekretieren. Das im Blut zirkulierende SEPP nimmt bei kritischen Erkrankungen, z.B. der Sepsis ab und führt zu erniedrigten Selenspiegeln, welche wiederum mit ansteigender Mortalität assoziiert sind. Sepsis triggert die übermäßige Produktion von proinflammatorischen Zytokinen einschließlich Interleukin-6 (IL-6) und daraus resultierendem oxidativen Stress, Gewebeschädigung und Organversagen. Zur Infektionsbekämpfung wird bei schwerer Sepsis oft ein Aminoglykosid-Antibiotikum (AG) angewendet. AG induzieren Fehlinterpretationen der mRNA und insbesondere des Stoppcodons UGA. Eine Rekodierung des UGA-Codons und eine Selenoprotein-spezifische Sec-Insertionssequenz (SECIS-Element) innerhalb mRNA sind während der Selenoproteinbiosynthese unabdingbar.

Da die Leber das wichtigste Organ der Selenregulation ist, wurden in dieser Arbeit die molekularen Wechselwirkungen zwischen proinflammatorischen Zytokinen (IL-6, Interleukin-1β und Tumornekrosefaktor-α), AG (G418 und Gentamycin) und dem Selenstatus mit der Selenoproteinbiosynthese in Hepatozyten untersucht.

IL-6 führte zu einer starken Reduktion der SEPP-mRNA und einer IL-6 dosisabhängigen Sekretion von SEPP. Parallel dazu reduzierte IL-6 das Transkriptlevel, die Proteinexpression und die Enzymaktivität des Selenoenzyms Jodthyronin-Dejodase Typ 1 (DIO1). Die Wirkungen von IL-6 auf die Expression der antioxidativ-wirkenden Glutathionperoxidasen (GPX) waren isozymspezifisch; während die Transkriptkonzentrationen von *GPX2* anstiegen und die von *GPX4* abnahmen, blieb *GPX1* unbeeinflusst. Diese IL-6-abhängigen Effekte spiegelten sich auch in Reportergenexperimenten von *SEPP-, DIO1-, GPX2*- und *GPX4*-Promotorenkonstrukten wider und weisen auf eine direkte Transkriptionsregulation durch IL-6 hin. Eine Kombination von IL-6, Interleukin-1β und des Tumornekrosefaktor-α führte zu einer stärkeren Abnahme der SEPP- und DIO1-Expression, sowie andererseits zu einer stärkeren Induktion der GPX-Enzymaktivität als durch IL-6 allein. Diese Ergebnisse weisen auf eine Umverteilung der Selenoprotein-Expression zugunsten von Selenoproteinen mit hoher Bedeutung bei entzündlichen Erkrankungen hin.

Um die Wirkungen von AG auf die Selenoprotein-Translation besser zu verstehen, wurden die SECIS-Elemente von *GPX1-, GPX4-* und *SEPP*-Transkripten in ein Reportersystem kloniert und auf eine Regulation durch AG und Se analysiert. Die Ergebnisse zeigen, dass der korrekte kotranslationale Einbau von Sec vom Selenstatus, von der AG-Konzentration und dem spezifischen SECIS-Element abhängig ist. Auf transkriptionaler und translationaler Ebene führten AG zu einem stark erhöhten SEPP-Spiegel, während die Expression und Enzymaktivität von GPX1, GPX2, GPX4 und DIO1 nur in geringerem Ausmaß beeinflusst wurden. Eine Analyse mittels Totalreflexions-Röntgenfluoreszenz zeigte, dass der Se-Gehalt von SEPP signifikant durch AG reduziert und vom Se-Status abhängig war. Insbesondere die Bedeutung des Selenstatus zur Überwindung der störenden und unterdrückenden Wirkungen von AG und entzündungsfördernden Zytokinen ist von hoher klinischer Relevanz. Es hebt Selenmangel als zentralen Risikofaktor für mögliche Nebenwirkungen hervor und verdeutlicht die Bedeutung einer ausreichenden Selengabe als sinnvolle Interventionsstrategie bei kritischen Erkrankungen.

1. Introduction

1.1. The trace element Selenium

1.1.1. History of Selenium

Selenium (Se) is a double-edged sword, an essential trace element and a poisonous substance at the same time. In early studies, only the toxic properties had been linked to Se, while its positive attributes remained hidden for centuries. In 1937, *Moxon et al.* published a report in which the toxic attribute of Se in livestock-poisoning plants was identified [Oldfield, 2002]. Presenting as hoof injuries in affected animals, this phenomenon was incorrectly named “Alkali disease”. The symptoms were later attributed to excessive Se-accumulation in the fodder plants of the affected livestock [Beath, 1935]. In 1957, the view to Se changed towards more constructive roles in organisms. It was at this time, the German biochemist Klaus Schwarz was investigating the origin of liver necrosis induced in laboratory rats fed on a diet were *Torula utilis* yeast was the protein source. When the researchers replaced the protein source with *Sacharomyces cerevisiae,* the symptoms of liver necrosis vanished. Studies of both yeasts revealed that *Sacharomyces cerevisiae* contained Se as opposed to *Torula utilis*, which did not. This led to the first identification of a Se-deficiency associated disease in animals [Schwarz, 1957]. Another milestone was the description of the first Se-containing protein in 1969, glutathione peroxidase [Flohe, et al., 1973; Rotruck, et al., 1973]. This discovery opened new avenues towards a better understanding of the biological role of Se.

1.1.2. Selenium metabolism in mammals

The intake of Se occurs almost exclusively via daily nutrition. Although various selenocompounds are found in the diet, almost all are in the form of selenomethionine (SeMet), selenocysteine (Sec), selenate, or selenite. SeMet is a Se-containing analogue of the amino acid methionine and is synthesised by plants. The incorporation of SeMet into proteins occurs randomly as an alternative to methionine. In some plants, ~90% of Se is in the form of SeMet [Cubadda, et al., 2010]. In animals, SeMet intake occurs via vegetable nutrition [McConnell and Cho, 1967]. Sec is synthesised in mammals and to a lesser extent by plants as an intermediate compound in the reverse transsulfuration pathway [Burk and Hill, 2015; Sors, et al., 2005]. In addition to the *de novo* synthesis, Sec can be taken up via daily nutrition.

Regardless of the form of the selenocompound, Se is readily absorbed via the lower small intestine (Figure 1). SeMet and Sec are resorbed via amino acid transporters [McConnell and

Cho, 1967]. The absorption of selenate occurs actively via a sodium-mediated carrier transport mechanism, while selenite diffuses passively [Fairweather-Tait, 1997].

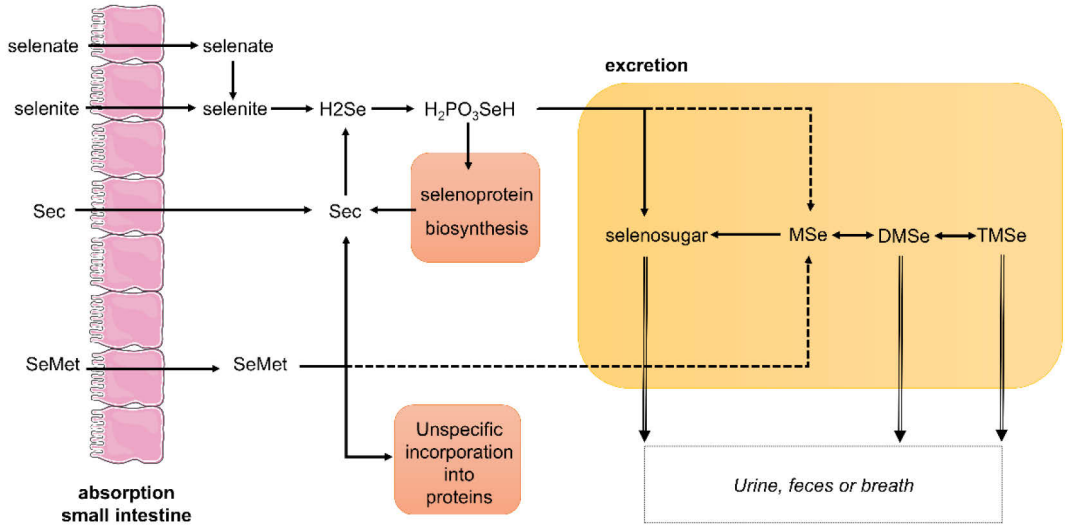


Figure 1: Se-metabolism in the human body. The small intestine mainly absorbs the selenocompounds SeMet, Sec, selenite or selenite. Selenate becomes directly reduced to selenite. SeMet is randomly incorporated into proteins, metabolised to Sec via the transsulfuration pathway or methylated for excretion. Sec and selenite are metabolised to selenide (H_2Se) and further to mono-selenophosphate (H_2PO_3SeH) by selenophosphate synthetase 2 which may enter either the selenoprotein biosynthesis or the excretion pathway. To be excreted, Se becomes methylated (mono-(MSe), dimethyl selenide (DMSe) or tri-methyl selenonium (TMSe)) or conjugated with N-actetyl galactosamine, followed by the methylation to different selenosugar compounds. The excretion may then occur via urine, faeces or breath. Adapted from [Roman, et al., 2014] using Servier Medical Art.

The absorbed selenocompounds are then transported via the blood stream to the liver. In the liver, selenocompounds become reduced to selenide before entering a complex biosynthesis machinery, resulting in the incorporation of Se in the form of Sec into so-called selenoproteins (section 1.2.). Although Sec is resorbed from the diet, it cannot be directly incorporated into selenoproteins. The incorporation requires a conversion of Sec to selenide and alanine by selenocysteine lyase. The selenophosphate synthetase 2 which is itself a selenoprotein catalyses the conversion from selenide to selenophosphate (H_2PO_3SeH). Selenophosphate is then either incorporated into selenoproteins or eventually excreted [Burk and Hill, 2015].

SeMet has three possible fates: 1) reduction to Sec via the transsulfuration pathway, 2) unspecific incorporation into proteins, or 3) excretion. Similar to plants, the biosynthesis machinery of mammals is unable to distinguish between SeMet and its analogue methionine. SeMet is hence incorporated randomly into proteins [Reilly, 2006]. In this context, a ratio of one SeMet molecule per 1,1000 albumin molecules has been described in healthy humans

[Burk, et al., 2001]. As the intake of SeMet increases, the amount of randomly incorporated SeMet at methionine residues in newly synthesised proteins increases accordingly. The half-life of overall Se in the human body is about 100 days [Griffiths, et al., 1976]. However, its retention depends on the Se-status, the general health status, the form of Se ingested and the tissue where Se is stored.

The excretion of Se occurs though the kidney, the gastrointestinal tract, or expiration via the lungs. It may also be excreted via the sweat, hair or nails, but mostly via the renal pathway [Yang, et al., 1989]. Se in faeces consists largely of non-absorbed dietary Se, combined with Se from intestinal, pancreatic and biliary secretions [Levander and Baumann, 1966]. If selenoproteins are optimally expressed, a further increase in selenite or selenite intake results in an almost complete Se-excretion above this optimum Se-intake level. If the Se-intake increases to an unusually high concentration, it becomes excreted via the breath [McConnell and Roth, 1966]. Excretory forms of Se are dimethyl selenide in breath, trimethyl selenonium in urine and selenosugar (1beta-methylseleno-N-acetly-D-galactosamine) in urine and faeces [Kobayashi, et al., 2002; Palmer, et al., 1969; Suzuki, et al., 2010]. They are mainly produced from selenide in liver by sequential methylation or conjugation with N-acetyl galactosamine and subsequent methylation steps [Mozier, et al., 1988]. However, the major excretory form of Se are selenosugars [Burk and Hill, 2015].

1.1.3. Selenium and human health

The Se-range between levels of dietary deficiency (< 40 µg Se/day) and toxic levels (>400 µg Se/day) is rather narrow [WHO, 1996]. An optimal Se-supplementation is U-shaped and ranges between 80-120 µg/L Se, with border zones of 60-80 µg/L Se and 120-140 µg/L Se (Figure 2) [Duntas and Benvenga, 2015]. A serum Se-concentration below 60 µg/L Se increases the risk for diseases and seems to aggravate ailments such as inflammation, autoimmunity, cancer, infertility or Se-deficiency associated diseases. Concentrations above 140 µg/L increase the risk of hyperglycaemia, type 2 diabetes, hyperlipidaemia or atherosclerosis and may also result in Se-intoxication, also known as selenosis [Duntas and Benvenga, 2015; Rayman, 2012].

Two Se-deficiency associated diseases have been described in humans, namely Keshan-disease and Kashin-Beck disease. Keshan-disease is characterised by a cardiomyopathy with multiple foci of necrosis closely associated with a dietary deficiency of Se [Lei, et al., 2011] and the presence of coxsackievirus B3 [Beck, et al., 2003]. Crops in the patients diet have been shown to be exceptionally Se-deficient (<0.04 mg/kg of Se) and Se-intake was less than 12

µg/day (40 µg/day required) [Li, et al., 2013]. The Keshan-disease is more prominent in farming communities being more reliant to their Se-deficient environment and resulting in Se-deficient food. Thus far Keshan-disease has mostly afflicted children and women in the region of Keshan in China [Chen, 2012]

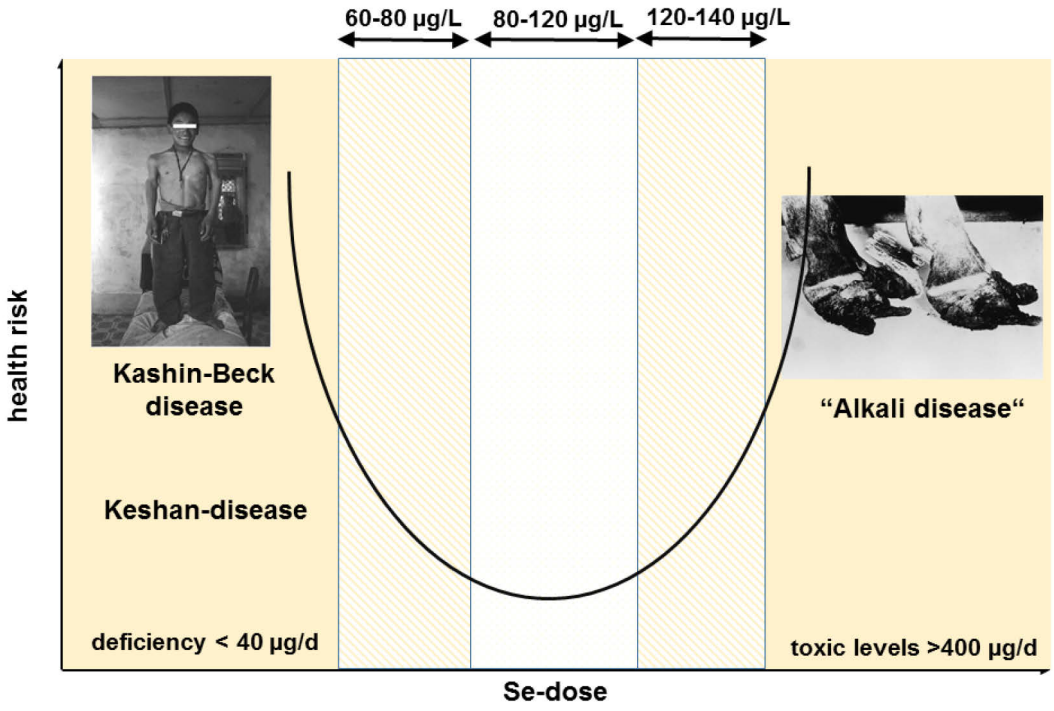


Figure 2: Se-intake and health risks.

Se-intake has a narrow range between dietary deficiency (< 40 µg/day) and toxic levels (> 400 µg/day). An optimal nutritional Se-intake ranges between 80-120 µg/L with a border zones of 60-80 and 120-140 µg/L Se. Serum Se-concentrations below 60 µg/L increase the risk for inflammation, autoimmunity, cancer, infertility or Se-deficiency associated diseases (Keshan-disease and Kashin-Beck disease). Concentrations above 140 µg/L increase the risk for hyperglycaemia, type 2 diabetes, hyperlipidaemia or atherosclerosis and may result in Se-intoxication, also known as selenosis or "Alkali-disease". Adapted from [Duntas and Benvenga, 2015; Moreno-Reyes, et al., 2003; Oldfield, 2002]

The Kashin-Beck disease is an osteochondropathy disease that is associated with iodine- and Se-deficiency. Patients suffer from joint deformations that affect peripheral joints and the spine. They are typically of short stature as a result of multiple focal necrosis in the growth plates of the tubular bones [Allander, 1994]. Se-deficiency is also associated with iodine deficiency disorders goitre and cretinism [Fordyce, 2013]. In rats, Se-deficiency caused an inhibition in hepatic deiodination of the thyroid hormone thyroxine (T4) [Beckett, et al., 1987]. The selenoprotein family of iodothyronine deiodinases are essential to the thyroid hormone metabolism. Thus, Se-supplementation can protect against Hashimoto's thyroiditis and positively affect mild Graves' disease [Rayman, 2012].

Se is also important for male fertility. Low Se-levels reduce the activity of the selenoenzyme glutathione peroxidase 4 (GPX4) that is essential for spermatogenesis [Rayman, 2012] or may cause immotile and deformity of sperm [Hawkes and Turek, 2001]. The role of Se in cancer is controversial as Se is associated with carcinogenic and anti-carcinogenic properties. In animal studies, selenite and organic selenocompounds reduced the incidence of diverse tumours. Similarly, protective properties of Se have been described against bladder, colorectal, lung, and prostate cancer possibly due to Se's antioxidant properties and the inhibition of nucleic acid and protein synthesis that is important to tumour growth [Clark, et al., 1998; Rayman, 2012]. On the other hand, Se may promote cancer based on the pro-oxidant mutagenic and immunosuppressive action of some selenocompounds. Selenium sulphide has been linked to carcinogenic effects in animal studies and with potential to act as a human carcinogen [Fordyce, 2013].

Initial descriptions of Se-toxicity can date back as far as the travels of Marco Polo who described poisonous plants that have been later found to store toxic amounts of Se and "if eaten by horses causes the hoofs to drop of" [Mihajlovic, 1992]. Further descriptions of hoof disorders in livestock have been reported in Columbia in 1560 and South Dakota in the mid-19th century which became known as "Alkali-disease" [Reilly, 2006]. As aforementioned, "Alkali-disease" is characterised by hoof deformation, hair loss and hypochromic anaemia [Fordyce, 2013; Levander, 1986]). In the 1930's, "Alkali-disease" became known as selenium toxicosis (selenosis) [Fordyce, 2013; Oldfield, 2002]. Cases of selenosis in humans are rare. However, one case study has related the intake of nuts of the *Lecythis ollaria* tree grown in Se-rich areas of Venezuela can induce vomiting and diarrhoea followed by hair and nail loss and death of two-year-old boys [Muller and Desel, 2010]. Cases of intoxication have further been reported in the USA as a result of faulty and miscalculated Se-supplementation of tablets causing nausea, vomiting, abdominal pain, diarrhoea, hair loss, brittle nails and peripheral neuropathy [MacFarquhar, et al., 2010; Morris and Crane, 2013].

1.2. Selenoproteins

1.2.1. Selenoprotein classification and function

Se plays a fundamental role in the maintenance of immune-endocrinology, metabolic and cellular homeostasis, which is mediated by selenoproteins [Brown and Arthur, 2001]. The selenoprotein family is characterised by the incorporation of the 21st proteinogenic amino acid Sec into the growing peptide chain. Selenoproteins are widely spread through all domains of life, i.e., in eubacteria, archaea and eukarya [Labunskyy, et al., 2014] ranging from one selenoprotein as found in *Caenorhabditis elegans* [Taskov, et al., 2005] to 59 as found in *Aureococcus anophageffenes* [Gobler, et al., 2013]. It is interesting to note that selenoproteins are not expressed in fungi and some animal species (e.g. red flour beetle *Tribolium castaneum* and the silkworm *Bombyx mori*) [Labunskyy, et al., 2014; Lobanov, et al., 2008]. The human selenoproteome is composed of 25 selenoprotein genes including the Se-transporter Selenoprotein P (SEPP), the family of glutathione peroxidases (GPX), the family of thioredoxin reductases (TXNRD), the family of iodothyronine deiodinases (DIO) and other selenoproteins with partially unknown function.

Selenoprotein P

Selenoprotein P (known as SEPP, SelP, SEPP1 or SELENOP) is a plasma selenoprotein which circulates as two isoforms and acts as the main Se-transporter in the body. In contrast to all other selenoproteins described, SEPP comprises some unique features, i.e., two SECIS-elements in the 3'untranslated region (UTR) of the mRNA and ten in-frame UGA codons allowing a maximal insertion of up to ten Sec-residues (Figure 3). The SEPP protein consists of two major domains, an N-terminal and a shorter Sec-rich C-terminal. The N-terminal domain carries one of the ten Sec-residues and a heparin-binding site that has peroxidase activity when bound to TXNRD1 [Kurokawa, et al., 2014]. The C-terminal domain possesses the remaining nine Sec-residues and is thus implicated in the Se-transport. The two SECIS-elements (SECIS1 and SECIS2) in the 3'UTR of the transcript have a different function with respect to supporting Sec-insertion in response to different UGA codons as described in section 1.2.2 [Stoytcheva, et al., 2006].

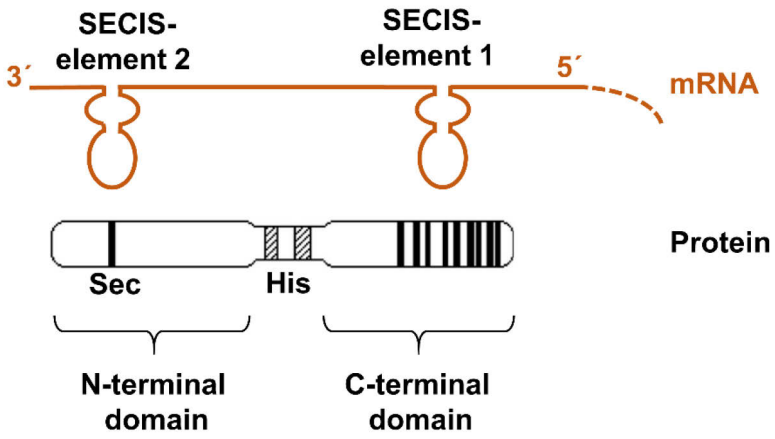


Figure 3: Schematic overview of Sec-insertion into SEPP.

The SEPP protein consists of two domains, an N-terminal and a shorter Sec-rich C-terminal. The N-terminal domain contains the first Sec-residue, whereas the C-terminal domain encompasses the second to the tenth Sec-residue. Two Sec-insertion sequence (SECIS)-elements, namely SECIS1 and SECIS2 are found in the 3'UTR of the *SEPP* mRNA. In a co-translational process, the SECIS-element, along with additional binding factors, mediates the Sec-incorporation at the side of the UGA codon. SECIS1 is required for the C-terminal, SECIS2 for the N-terminal insertion of Sec-residues. The N- and C-terminal domains are separated via two histidine (His)-rich areas. Modified from [Saito, et al., 2004].

SECIS2 is mandatory for the insertion of the first Sec-residue in the N-terminal domain, whereas SECIS1 is required for the insertion of the second to the tenth Sec-residue in the C-terminal domain. SECIS2-mediated Sec-incorporation is less efficient than SECIS1 [Berry, et al., 1993] resulting in a slower and potentially unsuccessful Sec-insertion at the first UGA codon. Once the ribosome reaches the second UGA codon, the SECIS2-mediated Sec-incorporation occurs rapidly allowing the insertion of the remaining Sec-residues in the shorter C-terminal domain [Burk and Hill, 2009].

90% of the synthesised SEPP is secreted by the liver [Burk and Hill, 2009; Hill, et al., 2012]. SEPP is then transported via the blood stream to peripheral Se-dependent organs including testes, kidney, brain or bone. At these peripheral organs SEPP is taken up by a member of the low-density lipoprotein receptor-related family, i.e., apolipoprotein E receptor-2 (apoER2 or LRP8) or megalin (LRP2) via endocytosis [Olson, et al., 2008; Olson, et al., 2007]. These receptors have different SEPP binding properties and different tissue expression pattern. LRP2 interacts with the N-terminal domain of SEPP thereby allowing the uptake of smaller SEPP

isoforms e.g. in the kidney [Kurokawa, et al., 2014]. The apoER2 receptor interacts with the C-terminal domain and is present at blood-brain barrier and in neurons [Burk, et al., 2014].

Glutathione peroxidases

The family of glutathione peroxidases (GPX) are widespread in all domains of life [Toppo, et al., 2008] and consists of eight isozymes of which five are selenoproteins: the cytosolic GPX (cGPX or GPX1), the gastrointestinal GPX (giGPX or GPX2), the plasma GPX (pGPX or GPX3), the phospholipid hydroperoxide GPX (PHGPX or GPX4) and olfactory GPX (GPX6). GPX are involved in hydrogen peroxide signalling, detoxification of hydroperoxides, and maintaining redox homeostasis. Hydrogen peroxide is an important signalling molecule that regulates a variety of processes and pathways, e.g. cell proliferation, apoptosis or stress response [D'Autreaux and Toledano, 2007], but can adversely induce oxidative tissue damage. Although all GPX catalyse the reduction of the hydrogen peroxide and alkyl hydroperoxides under the oxidation of glutathione, they markedly differ in their specificities for hydroperoxide [Brigelius-Flohe, 1999].

In 1973, *Rotruck et al.* and *Flohé et al.* described glutathione peroxidase (formerly GPX, now GPX1) as the first eukaryotic selenoprotein [Flohe, et al., 1973; Rotruck, et al., 1973]. GPX1 is expressed ubiquitously in all cell types showing the highest expression in liver and kidney. GPX1 catalyses degradation of soluble hydroperoxides, such as hydrogen peroxide or some organic hydroperoxides and thus prevents oxidative damage, lipid peroxidation and protein degradation. GPX1 belongs to the stress-related selenoproteins and is highly Se-sensitive [Baker, et al., 1993; Sunde, et al., 2009]. Its expression drops dramatically under Se-deficiency, especially in liver and kidney. GPX2 is mainly expressed in the epithelium of the gastrointestinal tract and is known to have anti-inflammatory and anti-carcinogenic properties [Brigelius-Flohe, 2006]. Its expression levels are negatively associated with tumour growth in different tissue types [Ewen and Hendry, 1990]. GPX3 is primarily expressed in the kidney and secreted into the plasma where it contributes to extracellular detoxification [Brigelius-Flohe, 1999]. GPX6 is expressed in the olfactory epithelium, and during embryogenesis [Kryukov, et al., 2003]. Interestingly, the Sec-residue in GPX6 is replaced by cysteine in some species, e.g. in rodents [Kryukov, et al., 2003]. GPX1, 2, 3 and 6 have a substrate specificity for hydrogen peroxide, and other soluble low-molecular weight hydroperoxides, e.g. tert-butyl hydroperoxide, cumene hydroperoxide, and short-chain fatty acid hydroperoxide, whereas GPX4 has substrate specificity for phospholipid hydroperoxides, e.g. phosphatidylcholine hydroperoxide or cholesterol hydroperoxide and other complex lipid hydroperoxides [Brigelius-

Flohe, 1999; Mates, 2000]. GPX4 is ubiquitously expressed during embryogenesis and in several adult tissues. In contrast to the Se-sensitive GPX1, GPX4 is less affected by the Se-status and belongs therefore to the housekeeping selenoproteins [Bermano, et al., 1995; Weiss Sachdev and Sunde, 2001]. GPX4 has three isoforms in which the mitochondrial and nuclear isoforms are only expressed in testes where it is essential for male gametogenesis [Schneider, et al., 2009].

Thioredoxin reductases

Thioredoxin reductases (TXNRD) catalyse the NADPH-dependent reduction of the redox protein thioredoxin (TRX). TRX acts as an antioxidant by reducing other proteins including peroxidases and ribonucleotide reductases and thus controls cellular redox state and protects against oxidative damage [Arner and Holmgren, 2000]. TXNRD are able to catalyse the reduction of other endogenous and exogenous compounds including glutathione and glutaredoxin. The wide substrate specificity of TXNRD is enabled by a second redox-active site within the catalytic centre. Three isozymes of TXNRD have been described in mammals: the cytoplasmic thioredoxin reductase 1 (TR1 or TXNRD1) [Tamura and Stadtman, 1996], the mitochondrial thioredoxin 3 (TR3 or TXNRD2) [Miranda-Vizuete, et al., 1999] and thioredoxin reductase 2 (TR2 or TXNRD3) that is exclusively expressed in testes [Miranda-Vizuete, et al., 2004]. Thioredoxin 1 (TRX1) is involved in antioxidative defence, regulation of transcription factors and apoptosis [Arner and Holmgren, 2000]. It serves further as an electron donor for several redox-active enzymes and is the major substrate of TXNRD1. In addition to TRX1, TXNRD1 catalyses the reduction of other low-molecular weight compounds [Arner and Holmgren, 2000]. In 1999, *Sun et al.* revealed that the Sec-residue of TXNRD1 functions as a sensor for reactive oxygen species [Sun, et al., 1999]. It has been shown that TXNRD1 activates the p53 tumour suppressor [Merrill, et al., 1999], and it therefore implicated in cancer prevention [Selenius, et al., 2010]. Controversially, TXNRD1 plays a role in tumour growth due to the high susceptibility of cancer cells to oxidative stress [Mandal, et al., 2010]. Furthermore, the thioredoxin system plays an important role in the regulation of several transcription factors such as NF-κB or AP-1 via modulating the intracellular redox levels [Arner and Holmgren, 2000].

Iodothyronine deiodinases

The family of iodothyronine deiodinases (DIO) is composed of: type I iodothyronine deiodinase (DIO1), type II iodothyronine deiodinase (DIO2) and type III iodothyronine deiodinase (DIO3). DIO are involved in the regulation of thyroid hormone (TH) activity by reductive deiodination. TH are involved in a diversity of processes during developing and in the adult organism, e.g. increasing cardiac output, heart rate, ventilation rate, and basal metabolic rate. The majority of the TH effects are mediated by nuclear TH receptors that have a high affinity for 3,3',5 triiodothyronine (T3) [Darras and Van Herck, 2012]. However, the thyroid gland produces primarily the biological inactive thyroid prohormone thyroxine (T4). The inactive prohormone T4 becomes activated by a 5'-deiodination reaction at the phenolic ring. This deiodination can be catalysed by DIO1 or DIO2 and results in the active T3 [Bianco, et al., 2002]. While DIO2 solely catalyses the deiodination at the phenolic ring, DIO3 exclusively targets the tyrosyl ring. DIO1 is the only DIO isozyme that catalyses both the phenolic and tyrosyl ring deiodination. With its specificity DIO3 is able to inactive both T3 and T4 to generate 3,3' T2 or reverse T3 (rT3). It is assumed that the circulating concentrations of TH are primarily regulated by DIO1 with a fine tuning of local T3 levels by DIO2 and DIO3 in a tissue-specific manner [Gereben, et al., 2008]. The local fine tuning is important for tissue regeneration after injury or tissue development, e.g. endochondral bone formation [Adams, et al., 2007]. The activity of DIO2 increases in muscle after injury and is associated with enhanced transcription of T3-dependent genes required for muscle differentiation and regeneration [Dentice, et al., 2010].

Table 1: Enzymatic function and expression pattern of the human selenoproteins

[Wrobel, et al., 2016].

Selenoprotein	Abbreviation	Function	Tissue
Glutathione peroxidases	*GPX*		
Cytosolic GPX	GPX1	Detoxification of hydrogen peroxide	ubiquitous
Gastrointestinal GPX	GPX2	Detoxification of hydrogen peroxide	epithelium of intestine
Extracellular GPX	GPX3	Detoxification of hydrogen peroxide	secretion from kidney to plasma
Phospholipid hydroperoxide GPX	GPX4	Inhibition of lipid peroxidation	wide expression range, testes
Glutathione peroxidase *6*	GPX6	Detoxification of hydrogen peroxide	olfactory epithelium

Thioredoxin reductases	***TXNRD***		
Cytosolic TXNRD	TXNDR1	Reduction of the oxidized form of cytosolic thioredoxin	ubiquitous
Mitochondrial TXNRD	TXNRD2	Formation/isomerization of disulphide bonds during sperm maturation	liver, kidney, heart
TR2	TXNRD3	Reduction of mitochondrial thioredoxin and glutaredoxin 2	testes
Iodothyronine deiodinases	*DIO*		
Type I DIO	DIO1	Deiodination of T4 to T3 or to rT3, and of T3 or rT3 to T2	thyroid gland, liver, kidney, pituitary
Type II DIO	DIO2	Deiodination of T4 to T3	thyroid, brain, muscle, heart
Type III DIO	DIO3	Deiodination of T4 to rT3 and T3 to T2	Brain, muscle, placenta
Other selenoproteins			
Selenoprotein H	SELH	Regulation of GSH synthesis and phase II detoxification enzymes	ubiquitous
Selenoprotein I	SELI	unknown	ubiquitous
Selenoprotein K	SELK	ER-associated degradation of misfolded proteins	heart, spleen, testes
Selenoprotein M	SELM	Rearrangement of disulphide bonds in the ER-localized proteins	brain
Selenoprotein N	SELN	Regulation of intracellular calcium mobilization	mainly muscle
Selenoprotein O	SELO	unknown	unknown
Selenoprotein P	SEPP	Se transport, antioxidant function	liver, brain, etc.
Selenoprotein R	SELR	Repair of oxidized methionine in proteins	mainly liver and kidney
Selenoprotein S	SELS	ER-associated degradation of misfolded proteins	ubiquitous
Selenoprotein T	SELT	Regulation of pancreatic b-cell function and glucose homeostasis	ubiquitous
Selenoprotein V	SELV	unknown	testes
Selenoprotein W	SELW	Redox regulation of 14-3-3 protein	ubiquitous
Selenophosphate synthetase 2	SPS2	Synthesis of selenophosphate	ubiquitous
15 kDa selenoprotein	SEP15	Quality control of protein folding	mainly kidney and liver

Other selenoproteins

Ten other selenoproteins with partially known functions have been identified in humans. Out of these ten, seven selenoproteins are located in the endoplasmic reticulum (ER). These proteins are involved in protein folding, maturation, quality control and cytokine response to stress or ER-stress regulation [Shchedrina, et al., 2010]. Selenoprotein S (SELS) for example contributes in the processing and removal of misfolded proteins from the ER into the cytosol. Translocated, misfolded proteins become polyubiquitinated and then degraded by the proteasome [Ye, et al., 2004]. SELS expression is activated according to ER stress or NF-κB signalling. Moreover, it plays an essential role in the production of inflammatory cytokines [Gao, et al., 2006]. Selenoproteins are further involved in the synthesis of selenophosphate or regulation of intracellular calcium mobilisation [Wrobel, et al., 2016].

1.2.2. Selenoprotein biosynthesis

Selenoproteins are characterised by the co-translational insertion of the 21st proteinogenic amino acid Sec. The Sec-incorporation into selenoproteins is highly regulated by a multistep biosynthesis machinery requiring a specifically modified Sec-tRNA$^{[Ser]Sec}$, an in-frame UGA codon, a selenoprotein specific stem-loop structure within the 3'UTR of the mRNA, the Sec-insertion sequence (SECIS)-element, and several selenoprotein synthesis specific biosynthesis proteins.

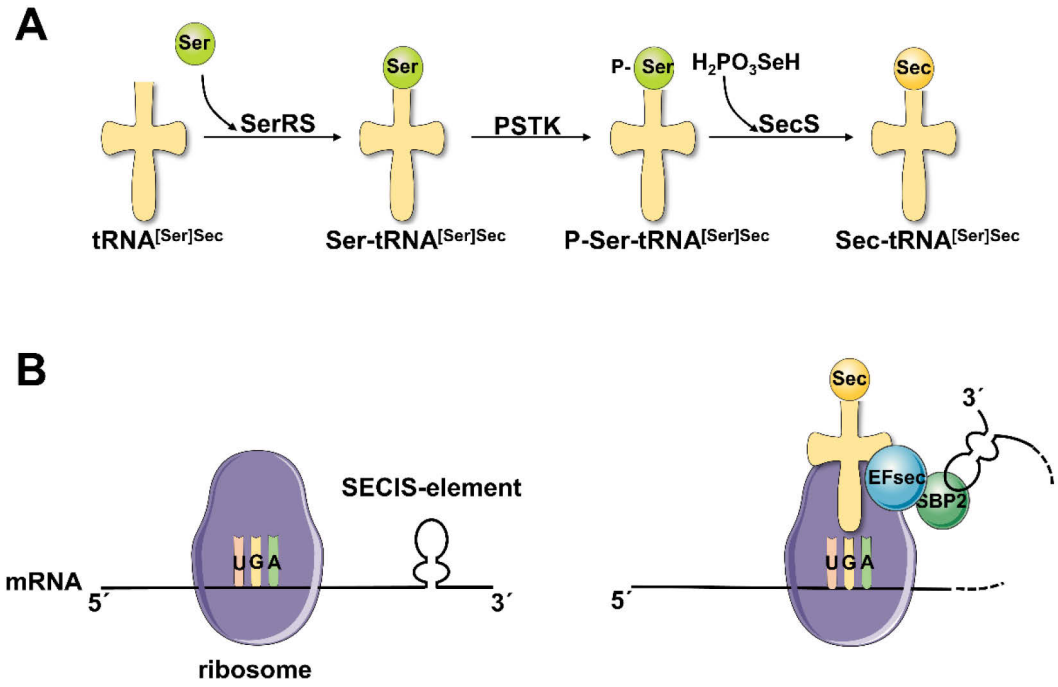


Figure 4: The selenoprotein biosynthesis machinery.

(A) Charging of Sec-tRNA$^{[Ser]Sec}$: The tRNA$^{[Ser]Sec}$ is charged with serine (Ser) by seryl-tRNA$^{(Ser/Sec)}$ synthetase (SerRS) to generate seryl-tRNA. The seryl residue becomes phosphorylated by O-phosphoseryl-tRNA$^{(Sec)}$ kinase (PSTK). Monoselenophosphate acts as Se-donor and is metabolised by selenocysteinyl-tRNA$^{(Sec)}$ synthetase (SecS) to yield Sec-tRNA$^{[Ser]Sec}$. (B) Sec-incorporation: The mRNA of selenoproteins contains an in-frame UGA stop codon and hairpin-structured Sec-insertions sequence (SECIS)-element in the 3'UTR. The Sec-specific elongation factor (EFsec) binds to the Sec-tRNA$^{[Ser]Sec}$ and the SECIS-element interacts with the SECIS-binding protein 2 (SBP2). When the UGA is recognised by the ribosome, EFsec interacts with SBP2 and induces Sec-incorporation into the growing peptide chain. Figure produced using Servier Medical Art.

The selenoprotein biosynthesis is initiated by the charging of the selenoprotein specific tRNA Sec-tRNA$^{[Ser]Sec}$ (Figure 4 A). The tRNA$^{[Ser]Sec}$ is first loaded with the amino acid serine (Ser). This charging is catalysed by the seryl-tRNA$^{(Ser/Sec)}$ synthetase (SerRS). The seryl-residue becomes then phosphorylated by the O-phosphoseryl-tRNA$^{(Sec)}$ kinase (PSTK). In the last step, the selenocysteinyl-tRNA$^{(Sec)}$ synthase (SecS) catalyses the replacement of the phosphoryl group by the highly active monoselenophosphate, metabolised from different

selenocompounds including dietary Sec (Figure 1), to yield selenocysteyl-tRNA (Sec-tRNA$^{[Ser]Sec}$) [Allmang and Krol, 2006].

The principle of Sec-incorporation requires the recoding of the stop codon UGA (Figure 4 B) with the help of the SECIS-element within the 3'UTR of the mRNA. The presence of the SECIS-element itself does not necessarily lead to Sec-incorporation. The SECIS-element is recognised by the SECIS-binding protein 2 (SBP2). In parallel, the Sec-specific elongation factor (EFsec) binds to the Sec-tRNA$^{[Ser]Sec}$. If an in-frame UGA codon is recognised by the ribosome, the loaded EFsec interacts with the SBP2. The formed Sec-insertion complex enters the ribosome and leads to Sec-incorporation into the growing peptide chain [Allmang and Krol, 2006].

1.2.3. Hierarchy of selenoproteins

The concept of selenoprotein hierarchy postulates that changes in the Se-status affect the synthesis of selenoproteins to a different and protein-specific degree. As Se is the limiting factor of selenoprotein biosynthesis, the expression of some selenoproteins becomes dramatically down-regulated in order to guarantee the full expression of others. This hierarchical concept is accomplished by different cis-acting factors, i.e., the UGA context, the SECIS-element and trans-acting factors, i.e., tRNA$^{[Ser]Sec}$, SBP2 or EFsec. Ribosomal profiling has revealed that Se-deficiency inhibits the UGA readthrough efficiency of some selenoproteins more intensively than others [Howard, et al., 2013]. The UGA codon context, also known as Sec-redefinition element (SRE), modulates the Sec-insertion efficiency albeit its mechanism is yet not fully understood [Howard, et al., 2007]. Additionally, the SECIS-element which helps in recoding the UGA codon shares some similarities (e.g. basic structure composed of two loops, conserved SECIS-core containing four non-Watson–Crick base pairs), but also displays some selenoprotein transcript-specific structural properties (e.g. additional bulge in the apical loop) [Bulteau and Chavatte, 2015]. These structural differences affect the binding of trans-acting factors thereby modifying the translation efficiency. The transcripts of *GPX1*, *SELW* and *SELH* are the most sensitively affected mRNA [Howard, et al., 2013; Sunde, et al., 2009].

Se-deficiency promotes the targeting of mRNA of Se-sensitive, low hierarchic selenoprotein transcripts for degradation by nonsense-mediated decay (NMD). The NMD targets aberrant mRNA with premature termination codons, as they are present in selenoprotein transcripts in the form of in-frame UGA codons, in order to reduce errors in gene expression [Seyedali and Berry, 2014]. As a result, GPX1 ranks near the bottom in this hierarchy, together with SELH,

SELW and SEPX1 [Howard, et al., 2013]. While GPX2 and GPX4 have shown to rank higher, SEPP and DIO1-3 are positioned in the middle of the hierarchical order [Wingler, et al., 1999]. The hierarchy of selenoproteins extends to the preference of Se-supply and Se-retention for different organs, with a greater priority for brain and testes [Burk and Hill, 2015]. An additional factor that contributes to the selenoprotein hierarchy is the methylation status of the $tRNA^{[Ser]Sec}$ that strongly depends on the Se-status. The methylated isoform is predominantly expressed under high Se-supply [Diamond, et al., 1993]. Each isoform is preferred by different selenoproteins [Carlson, et al., 2007]. Conditions such as acute phase reaction and oxidative stress are likely to alter this selenoprotein hierarchy [Burk and Hill, 2015].

1.2.4. Biomarker of selenium status

A biomarker is defined as a biologically derived indicator of biologic or pathogenic processes, or of the pharmacologic response to therapeutic interventions [Biomarkers Definitions Working, 2001; Sunde, 2010]. In a recent review, *Sunde et al.* described the hierarchal expression of any informative biomarker at different levels of Se-exposure [Sunde, 2010]. Accordingly, Se-intake, tissue Se-concentrations, Se-function in form of selenoproteins and Se-excretion are informative parameters and may serve as biomarkers at different levels of Se-exposure. The Se-intake is determined via the amount of ingested Se-containing food, water or other supplements. The Se-metabolism in humans is more difficulty to study than in experimental animals, as SeMet can be excluded in animal studies and Se-forms in dietary products may vary regional [Burk and Hill, 2015].

Se in tissue can be determined from whole blood, plasma, serum, erythrocytes, buccal cells, lymphocytes, nail and hair. Hair and nails offer access to a long-term Se-status. Unregulated components introduce background noise for example, anti-dandruff shampoos containing selenium sulphide may adulterate the Se-status [Navarro-Alarcon and Cabrera-Vique, 2008]. Se-function is monitored in the form of selenoproteins in tissue or plasma as described below. Lastly, Se-excretion can be determined in urine in the form of total Se, selenosugar or methylated Se, in faeces in the form of total Se and in breath in the form of methylated selenide. The level of excreted Se gives conclusions about non-absorbed Se, non-retained Se or excessive Se-intake [Combs, 2015].

Serum Se is the most commonly used biomarker for Se-status and can be either monitored in the form of total Se or selenoproteins. In humans, GPX1 and the plasma selenoproteins GPX3 and SEPP are the most useful selenoproteins. GPX1 is highly sensitive to Se-deficiency and a drop in Se-status can rapidly be monitored by the *GPX1* mRNA isolated from buccal cells,

erythrocytes or lymphocytes [Combs, 2015]. The Se-concentration in buccal cells is significantly related to Se-intake [Combs, et al., 2011]. However, GPX3 and SEPP status can be easier determined from blood. GPX3 and SEPP concentrations fall dramatically with increasing Se-deficiency [Yang, et al., 1989]. *Hill et al.* estimated a Se-plasma level of 80 ng/mL when both plasma selenoproteins are maximally expressed, whereas GPX3 shows a maximum activity at lower plasma Se-concentration [Hill, et al., 1996]. SEPP is thus the better biomarker for Se-status, but any extra increase in Se-level may not be quantifiable using these selenoproteins. However, the Se-status in the plasma of Se-deficient subjects respond to Se-supplementation in ratio to the extent of supplementation [Xia, et al., 2005], whereupon the relationship of intake and plasma level also depends on the consumed selenocompound. E.g. inorganic Se produces only increase of 20% in individuals with Se-concentration > 70 µg/L [Broome, et al., 2004; Burk, et al., 2006], while SeMet and Sec increase Se-plasma level in a wider concentration range.

The Se-status in plasma may also comprise a genetic component. In this respect, the *GPX1* 679T/T allele is associated with increased cancer risk [Hu and Diamond, 2003; Ratnasinghe, et al., 2000], and the carriers show lower plasma Se-concentrations than the *GPX1* 679C/C carriers [Combs, et al., 2012]. Individuals with the *SEPP* 24731 A/A allele have up to 27% higher plasma SEPP-concentrations than *SEPP* 24731 G/A or G/G carriers [Combs, et al., 2011]. The plasma Se-concentrations vary also with gender, decline with age, with a marked reduction in smokers [Lloyd, et al., 1983], subjects with protein malnutrition [Mathias and Jackson, 1982] and inflammation [Maehira, et al., 2002]. Taken together, these factors are likely to alter the strong correlation between plasma SEPP- and Se-concentrations and make it therefore necessary to determine both biomarkers for the identification of health risk.

1.3. Selenium and selenoproteins in critical illness

1.3.1. Critical illness: Systemic inflammation and sepsis

The term critical illness is not easy to define, but comprehends to the greatest possible extent life-threatening diseases, e.g. cancer, severe trauma or severe infectious diseases such as sepsis. Sepsis is serious bloodstream infection that can quickly become life-threatening and is among one of the major causes of death in intensive care units. Sepsis mainly arises from bacterial, but also viral and fungal infections. These infections can emanate from skin, lungs, abdomen, urinary tract and/or medical invasive procedures, e.g. catheter [Hall, et al., 2011]. In the USA alone, over one million individuals are afflicted by sepsis. With a mortality rate of 28-50% annually, sepsis has a higher mortality rate than that of cancer or AIDS [Hall, et al., 2011]. Sepsis can strike anyone, but certain sections of society are especially vulnerable including the very young (< one year), the elderly (>75 years), frail people including subjects with impaired immune system, or after invasive procedures [Centre, 2016]. Although the health system has massively improved over the last decades, the incidence of sepsis is increasing partly due to an aging population, the increased longevity of people with chronic diseases and the spread of antibiotic-resistant organisms.

Based on the ACCP/SCCM consensus conference, sepsis is diagnosed when two of the following criteria are given (Table 2, 1), a-d) and a source of infection has been proven by laboratory evidence of inflammation (Table 2, 2).

Table 2: Diagnosis criteria for sepsis

1)	**Two of the following criteria:**
	a) fever (≥38°C) or hypothermia (≤36°C)
	b) tachycardia heart frequency ≥90 /min;
	c) tachypnea (frequency ≥20/min) or hyperventilation ($PaCO_2$ ≤4.3 kPa/ ≤33 mmHg)
	d) leucocytosis (≥12000/mm3) or leukopenia (≤4000/mm3) or ≥10% immature neutrophils in the haemogram [Bone, et al., 1992]
2)	**In combination with laboratory evidence of inflammation, i.e. elevated IL-6, CRP or PCT**

Clinical criteria include the determination of early markers of bacterial infection, e.g. elevation in C-reactive protein (CRP), procalcitonin (PCT) or the pro-inflammatory cytokine Interleukin-6 (IL-6). In case of a lack of infection, the patient is alternatively diagnosed with systemic inflammatory response syndrome (SIRS). However, an early clinical intervention is necessary and includes at first the treatment with broad-spectrum antibiotics. Many patients suffer from oxidative stress [Reddell and Cotton, 2012]. Oxidative stress is caused by reactive oxygen or nitrogen-oxygen species that trigger systemic inflammation and lead to mitochondrial

dysfunction, tissue injury, organ failure and death [Galley, 2011; Heyland and Dhaliwal, 2005; Jones and Heyland, 2008]. The classic sepsis treatment is supported by immune-enhancing diets and pharmaconutrition, with an administration of antioxidants and cofactors that are dosed separately from the standard nutritional requirement [Dupertuis, et al., 2009; Jones and Heyland, 2008]. Antioxidants require cofactors including zinc, iron, Se, vitamin C and E [Santora and Kozar, 2010]. Arginine for example serves as an enhancer for T-cell function and is an important substrate for nitric oxide production [Santora and Kozar, 2010]. Depleted levels of arginine lead to decreased T-cell function and increased risk of infection [Jones and Heyland, 2008]. Zinc plays a role in immune function, wound healing, superoxide dismutase and glutathione activity and thiol pool stabilization [Luo, et al., 2008]. Low serum zinc levels are linked to immune dysfunction, higher infection rates and increased mortality after infections [Heyland, et al., 2008]. Se-supplementation is thought to improve the clinical outcomes in clinical illness by decreasing infectious complications and organ dysfunction [Taylor and Krenitsky, 2010] as described in section 1.3.3.

1.3.2. Selenium and the immune system

Se has an important role in the innate and adaptive immune system. The activation of immune cells through cell surface or intracellular receptors can lead to high levels of reactive oxygen species (ROS) within minutes, which is often referred to as an oxidative burst [Huang, et al., 2012]. The generation of ROS by immune cells is connected with the killing of microbes by phagocytes. ROS, that are produced by macrophages and neutrophils, are essential for the oxidative destruction of phagocytosed pathogens and a fully functional immune system [Huang, et al., 2012]. In T-cells, higher dietary Se produces stronger oxidative burst in response to T-cell receptor stimulation [Hoffmann, et al., 2010].

Immune cells express many, but not all selenoproteins [Huang, et al., 2012]. In immune cells, selenoproteins regulate or are regulated by cellular redox level, which is a crucial modulator of immune cell signalling, or carry out quality control of protein folding [Hoffmann, et al., 2007]. Immune cells do not differ much from other cell types in their selenoprotein expression pattern [Huang, et al., 2012]. In mouse spleen, *Gpx1, Gpx4, SelW, SelK* and *Sep15* belong to the most prominent selenoprotein transcripts [Hoffmann, et al., 2007], whereas in murine macrophages, *Gpx1, Gpx4, Sel15, Sepp; SelK, SelR* and *Txnrd1* are the most abundant transcripts [Carlson, et al., 2010]. Txnrd1 is particularly important to maintain the redox tone in immune cells and the most abundant selenoprotein in mouse macrophages [Carlson, et al., 2009]. SelK promotes the calcium flux that induces the activation of several types of immune cells [Verma,

et al., 2011]. Immune cells respond to increasing Se-intake with increasing activity of GPX1 and GPX4, as shown in lymphocytes [Broome, et al., 2004].

1.3.3. Selenium and selenoproteins in inflammation

The importance of Se in the immune system is undeniable and Se-supplementation is incorporated into the clinical guidelines for sepsis treatment. Some, but not all, clinical trials have proven that supplemental Se improves the outcome of critically ill patients, but the best application form, most suitable selenocompound and the mechanisms of action are still under discussion. On the one hand, *Angstwurm et al.* showed a reduced mortality in Se-supplemented patients with severe sepsis or septic shock (significantly reduced 28-day mortality (to 42%, as compared to 57% in the placebo group) [Angstwurm, et al., 2007]. On the other hand, *Forceville et al*. demonstrated that high dose of Se achieved no difference in duration of mechanical ventilation, stay in the intensive care unit or mortality between the placebo and the treated group [Forceville, et al., 2007]. The recently published Cochrain review has analysed 16 different Se-supplementation studies for critical ill adults and illustrated that differences in the study outcome may result from variations in the form of the applied selenocompound, way of application (parenteral or enteral) and the dosage applied. A comparison between these studies is therefore difficult. The authors criticised most studies lack the quality of evidence and exhibit substantial bias [Allingstrup and Afshari, 2015]. Nevertheless, we have learned from such studies that serum Se-concentration negatively correlate with the severity of sepsis/SIRS and mortality risk.

The plasma Se-concentration decreases primarily due to a redistribution of Se from serum into body compartments via capillary leakage, which is one out of five signs of inflammation [Berger and Chiolero, 2007; Maehira, et al., 2002]. *Hawker et al.* were among the first reporting that the plasma Se-concentration drops in ICU patients compared to healthy controls [Hawker, et al., 1990]. *Forceville et al*. showed that septic shock patients exhibited a 40% decrease in Se-level and 0.7 µmol/L plasma Se was associated with a fourfold increase in mortality [Forceville, et al., 1998]. Further studies investigated the Se-status and plasma selenoproteins in critical ill patients in relation to mortality. *Manzanares et al*. showed that a drop in GPX3 activity correlated inversely with severity of sepsis and mortality [Manzanares, et al., 2009]. *Forceville et al*. highlighted that patients with septic shock or multi-organ failure had 70% lower SEPP levels than patients without SIRS, and that the plasma SEPP concentration was lower in non-survivors as compared to survivors, while GPX showed no difference [Forceville, et al., 2009]. The authors postulated that SEPP, rather than GPX, is a potential marker of septic shock and

related syndromes. However, these studies are limited to adults and appropriate studies for neonates reflecting a high risk-group are missing.

First insights into the complex molecular mechanism between selenoproteins and infection were shown in experimental animal studies. In this context, Lipopolysaccharide (LPS)-injection has proven as a suitable and well-defined form of a sepsis model in animal experiments. LPS is derived from the outer membrane of gram-negative bacteria. An LPS-injection into the target organism results in sepsis-like symptoms and an acute phase response that is accompanied with an increasing release of pro-inflammatory cytokines including IL-6, interleukin-1β (IL-1β) and tumour necrosis factor α (TNFα) (Figure 5) [Benatti and Pedersen, 2015; Buras, et al., 2005].

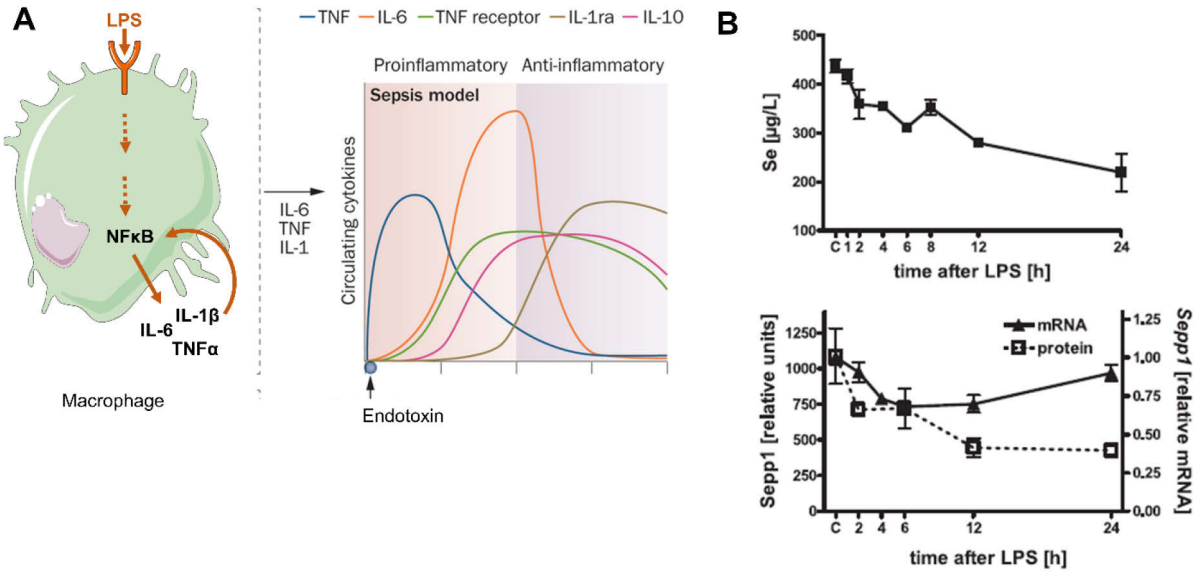


Figure 5: Se-metabolism and cytokine response in LPS-injected mice.

(A) In macrophages, the transcription of IL-6, TNFα and IL-1β is regulated by the Toll-like receptor signalling cascade and results in the activation of NF-κB after LPS exposure. A rapid increase in circulating TNFα occurs immediately after exposure to the endotoxin. The increase in TNFα is followed by a rise in IL-6 concentrations. Increasing cytokine levels lead to a negative feedback on the NF-κB activation. Modified and simplified from [Benatti and Pedersen, 2015] using Servier Medical Art. (B) LPS-injection results in a strong reduction of serum Se and Sepp concentration to 50% and 39%, respectively, whereas no significant drop in the *Sepp* transcript level was observed. Decrease in Se and Sepp in serum were proven to result from a decline of factors of the selenoprotein biosynthesis machinery [Renko, et al., 2009].

Taking advantage of this sepsis-model, preliminary data from our group have shown a strong down-regulation of the hepatic selenoprotein biosynthesis machinery and impaired Se-metabolism during the acute phase response in mice [Renko, et al., 2009]. Serum concentration of Se and Sepp declined in parallel after an LPS-injection, to 50% and 39%, respectively. While the mRNA of *Sepp* was not impaired by LPS-injection, a set of hepatic

transcripts involved in selenoprotein biosynthesis, e.g. EFsec, Sps2, SecS and most strongly PSTK declined coordinately during an acute phase response and contribute to the strong decline of *Sepp* [Renko, et al., 2009]. *Carslon et al.* further demonstrated that murine LPS-activated macrophages increase Txnrd1 expression at transcript and protein level [Carlson, et al., 2009]. Constitutive studies revealed a post-transcriptional, sex-specific up-regulation of the ER-stress regulator SelS during the acute phase response in mice and highlight the complexity of selenoprotein regulation in the acute phase response [Stoedter, et al., 2010].

1.3.4. Selenoproteins and pro-inflammatory cytokines

Sepsis is characterised by an imbalance of pro- and anti-inflammatory cytokines with an increased shift towards the pro-inflammatory cytokines. The major pro-inflammatory cytokines that regulate an early immune response comprise IL-6, IL-1β and TNFα [Chaudhry, et al., 2013]. They act as endogenous pyrogens by up-regulating the synthesis of secondary mediators of inflammation, by the up-regulation of other pro-inflammatory cytokines by macrophages or mesenchymal cells and by stimulating the production of acute phase proteins by the liver, e.g. ceruloplasmin or C-reactive protein [Chaudhry, et al., 2013]. The liver does not only produce a large number of acute phase proteins, but also expresses numerous selenoproteins including the Se-transporter SEPP and thereby contributes to controlling systemic Se-metabolism (Figure 6). IL-6 is secreted by T-cells and macrophages to stimulate the immune response to trauma or in response to specific microbial molecules. Plasma IL-6 levels are elevated in patients with sepsis [Gouel-Cheron, et al., 2012], and even higher levels are observed in patients with septic shock or who have died from severe sepsis [Wu, et al., 2009]. High IL-6 concentrations are associated with highest risk of death in patients with sepsis [Kellum, et al., 2007] and correlate to mortality rate in patients suffering from sepsis [Kumar, et al., 2009]. IL-1β is another mediator of the inflammatory response. It is involved in cell proliferation, differentiation and apoptosis. Patients that died from sepsis consistently showed higher serum IL-1β concentrations [Mera, et al., 2011]. TNFα induces apoptotic cell death and inhibits viral replication [Wallach, 1997]. In parallel to IL-6 and IL-1β, the TNFα serum concentration is significantly increased in patients with sepsis and in animal models [Mera, et al., 2011]. Independent from an impaired selenoprotein biosynthesis machinery during the acute phase response, a negative impact of IL-6, IL-1β and TNFα on selenoprotein expression has been reported in several publications.

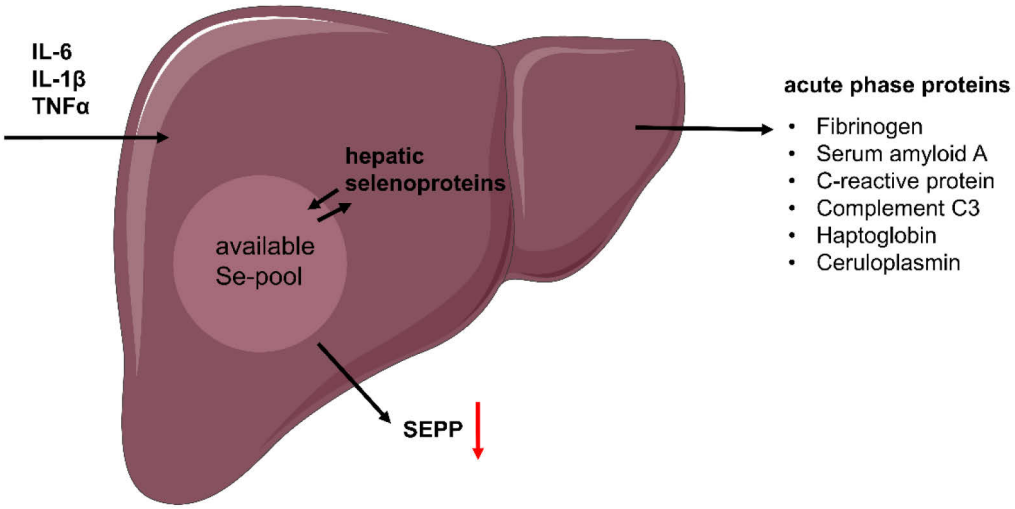


Figure 6: Hepatic acute phase proteins and selenoproteins.

The pro-inflammatory cytokines promote the hepatic production of many acute phase proteins, e.g. Fibrinogen, Serum amyloid A, C-reactive protein, Complement C3, Haptoglobin and Ceruloplasmin. These cytokines also regulate the expression of hepatic selenoproteins and the Se-transporter SEPP thereby affecting overall Se-metabolism. Figure produced using Servier Medical Art.

Promoter studies of SEPP indicate an interaction with cytokine and growth factors, repressing the *SEPP* promoter activity and thereby SEPP expression [Al-Taie, et al., 2002; Dreher, et al., 1997; Mostert, et al., 2001]. *Speckmann et al*. highlighted that the individual cytokines IL-1β, interferon-γ and TNFα reduce protein and transcript levels of SEPP to a minor extent, whereas a combination of these cytokines cause an over 50% SEPP reduction in protein and transcript levels, associated with a reduced SEPP promoter activity in the colorectal adenocarcinoma cells Caco-2 [Speckmann, et al., 2010]. The authors further concluded that the down-regulation of the intestinal SEPP expression occurs via the induction of nitric oxide synthase 2 and that this effect may contribute to the emergence of inflammatory bowel disease-related colorectal cancer [Speckmann, et al., 2010]. In this context, GPX2 is highly expressed during inflammatory bowel disease and colorectal cancer. Subsequent animal studies in mice identified Gpx2 as a novel target of *signal transducer and activator of transcription* (STAT) transcription factors including STAT3. STAT3 is underlying the IL-6 signalling pathway [Hiller, et al., 2015]. Polymorphism studies in 522 individuals from 92 families identified associations between *SELS* polymorphisms and IL-6, IL-1β and TNFα concentrations. Interestingly, the suppression of SELS by short interfering RNA in macrophage cells increased the release of IL-6 and TNF-alpha, indicating that selenoproteins are not only regulated by cytokines, but also

vice versa [Curran, et al., 2005]. IL-1β and TNFα have also demonstrated to upregulate SelS via the NF-κB pathway, which might serve as evidence for a regulatory loop [Gao, et al., 2006]. However, there are still many regulatory relationships between cytokines and selenoprotein biosynthesis that require further decryption.

1.3.5. Selenoproteins and aminoglycoside antibiotics

An early application of a broadband antibiotic is the strongest weapon to combat sepsis. There are several different classes of suitable antibiotics of which some, e.g. the aminoglycosides (AG) are used less frequently due to their broad negative side effects. However, the rise of resistance to multiple antibiotics revived the application of this class of antibiotics. AG belong to the protein synthesis inhibitors whose clinical application is limited to serious cases, e.g. systemic infections like severe sepsis of neonates or for local severe infections [Tsunemoto, 1987]. Endogenous mechanisms for AG degradation are missing in humans and the substances are thus excreted via the kidney [Gonzalez and Spencer, 1998]. AG may nevertheless accumulate in the kidney cortex and structures of the inner ear leading to nephro- and ototoxicity in treated individuals [Kent, et al., 2014]. The application period is hence strongly limited and AG serum concentrations are tightly controlled. Most antibiotics cover a wide application rage, whereas the use of AG is mainly restricted to gram-negative, aerobic bacteria. As AG cannot be absorbed by the intestine, they have to be applied intravenously [Gonzalez and Spencer, 1998].

AG interfere with the small 30S ribosomal subunit of bacteria, but also with the small 40S subunit of eukaryotes. This interference impairs the proofreading process and leads to the misinterpretation of codon [Poulikakos and Falagas, 2013; Ryu and Rando, 2001]. The misinterpretation leads either to the insertion of alternative amino acids or to a premature termination resulting in biosynthesis of truncated and/or non-functional proteins. AG may also induce the misinterpretation of the stop codons UAG (amber), UGG (ochre) and UGA (opal), promoting extended translation or suppressing premature stop codons [Keeling, et al., 2012]. Out of these three stop codons, the UGA codon is the most sensitive one [Kimura, et al., 2005]. Notably, the UGA codon is also coding for the insertion of Sec-residues into the growing peptide chain of selenoproteins (Figure 7 A). A misinterpretation of UGA codon may lead to the insertion of alternative amino acids and thus Sec-free, non-functional selenoproteins (Figure 7 B).

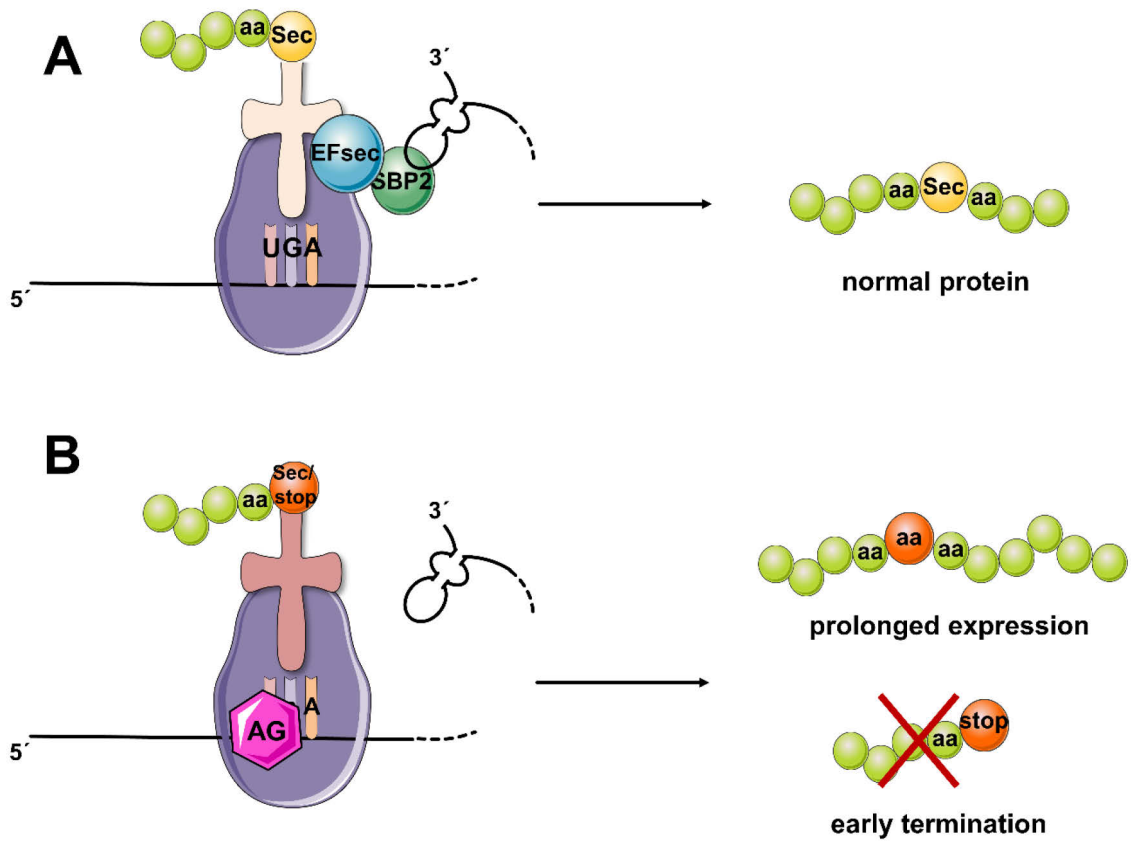


Figure 7: Interference of AG with selenoprotein biosynthesis.

(A) Functional selenoprotein biosynthesis requires the recoding of the stop codon UGA mandatory for the incorporation of Sec-residues. **(B)** Interference of AG with the small 40S ribosomal subunit of eukaryotes may lead to the misinterpretation of the UGA codon leading 1) to the insertion of alternative amino acids and thereby to Sec-free selenoproteins 2) to the expression of prolonged proteins. Abbreviation: aa= amino acid. Figure was created using Servier Medical Art.

So far, the effects of AG on selenoprotein expression have mainly focused on the application of geneticin (G418). G418 is structurally similar to the clinically used gentamicin. In a pioneering study, *Handy et al.* demonstrated that G418 increases the UGA readthrough in Se-replete cells, even in Se-absence, resulting in an increased immunodetectable, but enzymatically inactive variant of GPX1 with a substitution of L-arginine for Sec [Handy, et al., 2006]. Studies of *Tobe et al.* have provided first comparative data on AG affecting selenoproteins differently due to varying degrees of error induction at UGA codons. The authors demonstrated a dose-dependent decrease in the enzymatic activity and Sec-insertion rate for TXNRD1, GPX1 and GPX4. The total Sec-amount in these selenoproteins varied strongly from as low as 30% in TXNRD1 to as >60% in GPX1 and GPX4. In line with the findings of *Handy et al.,* AG induced a substitution of preferably L-arginine for Sec in GPX1 and of L-arginine, cysteine and tryptophan for Sec in GPX4 [Tobe, et al., 2013]. An even more

complex interplay of selenoproteins and AG has been revealed by detailed cloning studies of the UGA codon context of the rat *Sepp* gene by *Grupta et al.* (2007). The authors showed significant differences spanning an 8-fold range of UGA readthrough efficiency resulting from different codon contexts. It is interesting to note that increasing concentrations of SBP2 strongly reduced UGA misinterpretation. Mutations of either of both SECIS-elements (SECIS1, SECIS2) of the *Sepp* transcript resulted in different effects of UGA readthrough. This gives a first evidence that UGA readthrough depends also on the particular SECIS-element [Gupta and Copeland, 2007]. Although the current literature regarding AG and selenoproteins is limited, it has uncovered a complex interplay involving the UGA context, the SECIS-element and the availability of SBP2.

1.3.6. Selenium and SEPP status in critical ill neonates

As neonates are lacking a distinctive immune system, they are much more sensitive to environmental factors such as infections, and thus belong to the high-risk group for sepsis. An early clinical intervention in the form of antibiotic application and stabilizing their Se-status is of particular importance in order to improve the outcome of infection and their survival rate. The Se-status in newborns strongly relies on the Se-intake of the mother either via umbilical cord transfer or breast milk feeding. However, the plasma Se-concentrations in newborns are lower compared to the Se-concentrations of their mothers, and even lower in preterm infants when compared to mature infants [Amin, et al., 1980; Darlow and Austin, 2003]. In very early preterm, a low Se-level is linked with an increased risk of chronic neonatal lung disease [Darlow and Austin, 2003; Lockitch, et al., 1989]. Although the Se- and SEPP plasma concentrations are noted to decrease in sepsis and negatively correlate with the severity of the disease in adults [Forceville, et al., 2009; Hollenbach, et al., 2008; Manzanares, et al., 2009], respective knowledge in newborns is currently unknown. *Darlow et al.* reported that lower plasma Se in infants is associated with increased respiratory morbidity [Darlow, et al., 2000]. We have recently investigated the Se, SEPP and IL-6 concentrations in the plasma of neonates with proven infection in order to identify a biomarker for overall Se-status in healthy and infected newborns [Wiehe, et al., 2016][1]. It was found that the plasma Se- and SEPP-concentrations negatively correlate with increasing IL-6 plasma concentrations (> 500 ng/mL IL-6) (Figure 8).

[1] To note, increasing plasma concentrations of IL-6 have the highest correlation to mortality rate in severe sepsis patients (Chaudhry et al., 2013).

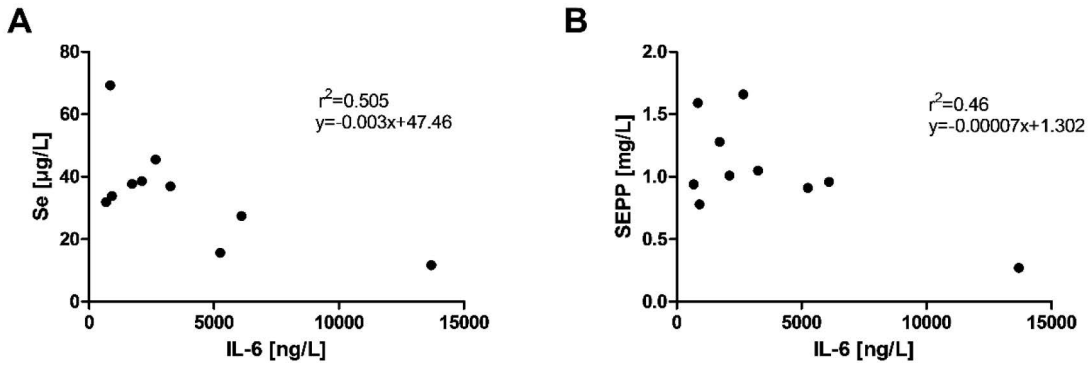


Figure 8: IL-6 and SEPP plasma concentrations in neonates with connatal infection.

Plasma concentrations of Se (A) and SEPP (B) negatively correlate in infected neonates with IL-6 serum concentrations > 500 ng/L. Adapted from [Wiehe, et al., 2016].

These findings are in line with a similar study of neonates with proven and clinical sepsis, where the plasma SEPP concentrations dropped from an average of 2.5 mg/L to under 1mg/L, and the IL-6 plasma concentrations increased from 60.1±7.8 pg/mL in healthy neonates to 90.8±2.9 pg/mL in infected newborns [Asci, et al., 2015]. Moreover, it was shown that the Se-concentrations in erythrocytes decreased in newborns with sepsis, however, to a lesser extent when compared to the drop in plasma SEPP-concentrations [Asci, et al., 2015]. These findings suggest that the correlation of decreasing Se-status with an increased severity of critical illness already exists in newborns.

In line with the *in vitro* studies, addressing the interplay of selenoproteins and aminoglycosides [Gupta and Copeland, 2007; Handy, et al., 2006; Tobe, et al., 2013], *Wiehe at al. (2016)* investigated whether an antibiotic therapy with the aminoglycoside gentamicin might alter the serum concentrations of Se and SEPP directly (Figure 9). Significant differences in the plasma SEPP concentrations before and after antibiotic treatment were found in the infected neonates. While the plasma SEPP concentrations increased significantly after treatment, the total plasma Se-concentration remained unaffected (Figure 9 A+B). The plasma Se-concentrations negatively correlated with increasing gentamicin concentrations, while no significant correlation was observed between gentamicin and SEPP (Figure 9 C+D) [Wiehe, et al., 2016]. The nature of these observations however, remained elusive.

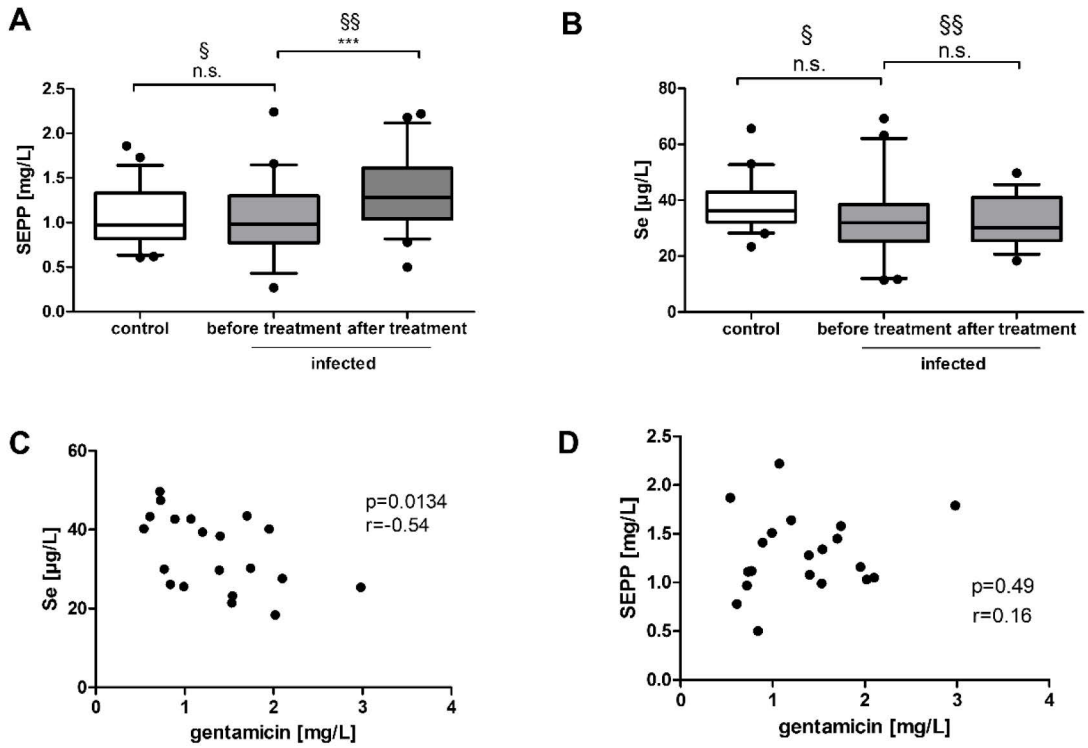


Figure 9: Se and SEPP serum concentrations in neonates with connatal infection.

Changes in the Se and SEPP plasma concentrations were measured before and after combined antibiotic treatment (ampicillin and gentamicin). (A) The Se-concentrations were decreased in the infected individuals compared to the control group, but showed no difference before and after treatment. The SEPP concentrations showed no difference between the group before treatment, but the plasma SEPP concentration significantly increased in the infected group after treatment. (C) The Se-concentrations negatively correlated with blood gentamicin concentrations, (D) while the SEPP concentrations were positively related to gentamicin concentrations (Legend: § paired t-test no normal distribution $$ paired t-test normal distribution). Adapted from [Wiehe, et al., 2016].

Based on the results of this study [Wiehe, et al., 2016], the questions arose whether IL-6 directly regulates SEPP and thereby affects other hepatic selenoproteins, and whether the observed increase in SEPP plasma concentrations resulted directly from gentamicin treatment.

1.4. Objective

In critical inflammatory diseases like sepsis, the plasma Se and SEPP concentrations decrease over time and inversely correlate with outcome and mortality risk. Clinical studies have indicated that Se-supplementation may improve this negative outcome. Sepsis is amongst the three main causes of death, as reported by Deutsche Sepsis-Hilfe e.V. As sepsis can become rapidly life-threatening, an early diagnosis, and timely clinical intervention is required. This includes the early application of broadband antibiotics, e.g. aminoglycoside antibiotics, in order to combat the infection efficiently. *In vitro* experiments have revealed that aminoglycosides interfere with the Se and selenoprotein metabolism. In serious cases, such as severe sepsis of neonates, the application of aminoglycosides, in particularly gentamicin, has been well established in the clinics. In a recently published study of newborns with connatal infection, we have shown that high IL-6 plasma concentrations, a clinical marker for sepsis that correlates with the mortality risk of sepsis patients, negatively correlates with decreasing Se and SEPP plasma concentrations. However, the SEPP plasma concentration increased in infected individuals after treatment with the aminoglycoside gentamicin. As SEPP is an important Se-supplier for many Se-dependent tissues, e.g. brain, testes and kidney, its down-regulation may lead to a reduced Se-supply in these organs, followed by a reduced expression of stress-related selenoproteins. Consequently, this may result in increasing ROS levels and increasing tissue damage in these organs and potentially organ failure

To this end, this thesis aimed to identify and characterise the regulation of SEPP and other hepatic selenoproteins by,

1. the pro-inflammatory cytokine IL-6 alone and in combination with IL-1β and TNFα
2. aminoglycoside antibiotics
3. IL-6 combined with aminoglycoside application

In Se-supplemented and Se-depleted liver cells, in order to convey potential therapeutic strategies to minimise negative effects and to contribute to an improved clinical outcome.

From this point, the following hypothesis was tested:

The hepatic selenoprotein expression is modulated by a complex interference of the pro-inflammatory cytokines IL-6, IL-1β and TNFα, aminoglycoside antibiotics and the actual Se-supply.

2. Material and Methods

2.1. Consumables

If not described differently all consumables were ordered from BD Biosciences (Heidelberg, Germany), Eppendorf AG (Hamburg, Germany) and Sarstedt AG & Co (Nümbrecht, Germany).

2.2. Chemicals

All chemicals were ordered from Roth (Karlsruhe, Germany), Sigma-Aldrich (Hamburg, Germany) and Merck (Darmstadt, Germany).

2.3. Solutions

All solutions are based on ddH_2O.

Table 3: Standard solutions

Name	Concentration	Component
20x PBS (pH 7.5)		
	2.75 M	NaCL
	54.05 M	KCL
	157.30 M	$Na_2HPO_4 \bullet 2H_2O$
	29.39 M	KH_2PO_4
6x Loading buffer for TAE (pH 7.5)		
	200 mM	TRIS-HCL
	50% v/v	Glycine
	4% w/w	SDS
	0.04% v/v	Bromophenol blue
	125 mM	DTT
Homogenisation buffer (pH 7.4)		
	250 mM	Sucrose
	20 mM	Hepes
	1 mM	EDTA (pH 8.0)

Table 4: Western blot solutions

Name	Concentration	Component
20% Gel solution		
	64.92% v/v	Gel solution A (30% acrylic amide)
	26% v/v	Gel solution B (2% Bisacrylic amide)
	9.08% v/v	ddH_2O
Separation gel buffer (pH 8.8)		
	1.5 mM	Tris base (MW 121.14 g/mol)
	0.4% w/w	SDS
Stacking gel buffer (pH 6.8)		
	0.5M	TRIS●HCL (pH 8.0)
	20% v/v	SDS
10x Running buffer for SDS-Page (pH 8.4)		
	192 mM	Glycerine
	25 mM	TRIS●HCL (pH 8.0)
	0.1% w/w	SDS
4x Loading buffer for SDS-Page (pH 7.5)		
	200 mM	TRIS●HCL (pH 8.0)
	50% v/v	Glycerine
	4% w/w	SDS
	0.04% v/v	Bromophenol blue
	125 mM	DTT
10x Transfer buffer		
	25 M	Tris base (MW 121.14 g/mol)
	192 M	Glycerine
	10% v/v	Methanol
10x TBS-Tween (pH 7.2-7.4)		
	0.2 M	Tris base (MW 121.14 g/mol)
	1.5 M	NaCL (MW 58.44 g/mol)
	0.05% v/v	Tween
Blocking solution		
	1% w/w	TBS-Tween
	5% w/w	Skim milk powder
20x Ponceau S stock solution		
	0.1% v/v	Ponceau S
	5% v/v	Acetic acid

Table 5: Buffers for enzyme activity assays

Name	Concentration	Component
GPX activity assay		
Basic buffer		
	100 mM	TRIS●HCL (pH 7.6)
	5 mM	EDTA (pH 8.0)
	1 mM	NaN_3
TXNRD activity assay		
1M Potassium phosphate buffer (pH 7.0)		
	61.5 % v/v	1 M K_2HPO_4 (MW 174.2)
	38.5% v/v	1 M KH_2PO_4 (MW 136.1)
DIO1 activity assay		
1 M Potassium phosphate buffer (pH 6.8)		
	49.7% v/v	1 M K_2HPO_4 (MW 174.2)
	50.3% v/v	1 M KH_2PO_4 (MW 136.1)
	10 mM	EDTA (pH 8.0)
Iodine-determination buffer 1		
	25% v/v	0.1 M Ammonium cerium (IV) sulphate solution
	65% v/v	UltraPure H_2O (Biochrom)
	1% v/v	5 M H_2SO_4
Iodine-determination buffer 2		
	50% v/v	0.05 M Sodium arsenide solution
	40% v/v	UltraPure H_2O (Biochrom)
	10% v/v	5 M H_2SO_4
	200 mM	NaCL

2.4. Commercially available systems

Table 6: List of ready-to-use kits

Ready-to-use kit	Provider
Absolute™ QPCR SYBR® Green Fluorescein Mix	Thermo Fisher Scientific, Winsford, UK
Amersham™ ECL™ Western Blot Detection Reagent	GE Healthcare UK Limited, Little Chalfont, UK
BCA™ Protein Assay Kit	Pierce, Rockford, USA
Dual-Luciferase®-Reporter Assay System	Promega, Mannheim, Germany
FastPasmid® Mini	Eppendorf, Hamburg, Germany
iScript™ cDNA Synthesis Kit	Bio-Rad Laboratories GmbH, Munich, Germany
KAPA HiFi HotStart ReadyMix PCR Kit	KAPA Biosystems, Boston, USA
Perfectprep® Gel Cleanup	Eppendorf, Hamburg, Germany
Pure Yield™ Plasmid Midiprep System	Promega, Mannheim, Germany
Selenotest ELISA	ICI immunochemical intelligence GmbH, Berlin, Germany
T4 DNA Ligase	New England Biolabs Inc., Frankfurt a.M., Germany
Renilla-Juice detection system	p.j.k GmbH, Kleinbittersdorf, Germany
Beetle-Juice detection system	p.j.k GmbH, Kleinbittersdorf, Germany

2.5. Enzymes, Cytokines, Aminoglycosides and Antibodies

Restriction enzymes

All restriction enzymes, BSA and the according buffers were ordered from New England Biolabs Inc. (Frankfurt a.M., Germany). For double digestion with different restriction enzymes, buffers were used according to Double Digest Finder software (New England Biolabs GmbH). The following restriction enzymes (20,000 units/mL) were used: EcoRI, EcoRV, HindIII, KpnI, NheI, SacI, and XhoI (New England Biolabs GmbH).

Cytokines

Table 7: List of pro-inflammatory cytokines

Cytokine	Source	Provider
IL-6, human recombinant	*E.coli*	Biomol GmbH, Hamburg, Germany
IL-1β, human recombinant	*E.coli*	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
TNFα, human recombinant	*E.coli*	Preprotech, Rocky Hill, United States

Aminoglycosides

Table 8: List of used antibiotics

Antibiotic	Company
G418 disulfate/geneticin	AppliChem GmbH, Darmstadt, Germany
Gentamicin solution	Sigma Aldrich, Hamburg, Germany
Amikacin	Fisher Scientific, Reinbach, Switzerland
Tobramycin	SERVA Electrophoresis GmbH, Heidelberg, Germany
Neomycin	Sigma Aldrich, Hamburg, Germany
Streptomycin	Fisher Scientific, Reinbach, Switzerland

Antibodies

For protein detection by Western blot or Dot blot analysis the following primary and secondary antibodies were used (Table 9).

Table 9: List of all antibodies used for Western blot and Dot blot

Name	Species	Purchase number	Dilution factor	Provider
Primary antibody				
anti-β-actin-Peroxidase	mouse	A3854	1:25,000	Sigma Aldrich, Hamburg, Germany
anti-Ceruloplasmin	goat	ab19171	1:2,000	Abcam®, Cambridge, UK
anti-DIO1 1068 (human, C-term)	rabbit	-	1:2,000	Mr. Kuiper via AG Schweizer, Institute for Experimental Endocrinology, Charité, Berlin
anti-SEPP (2B5-G5x2)	monoclonal, mouse	-	1:2,000	Invivo BioTech Services GmbH, Henningsdorf, Germany
anti-GPX1	rabbit	ab16798	1:1,000	Sigma Aldrich, Hamburg, Germany

anti-GPX2	rabbit	-	1:5,000	Prof. Kipp [Hiller, et al., 2015]
anti-GPX4	rabbit	ab40993	1:1,000	Sigma Aldrich, Hamburg, Germany
Secondary antibody				
anti-goat-IgG/HRP	rabbit	P0449	1:2,000	Dako Denmark A/S, Glostrup, Denmark
anti-rabbit-IgG/HRP	goat	P0448	1:2,000	Dako Denmark A/S, Glostrup, Denmark
anti-mouse-IgG/HRP	sheep	NXA931	1:2,500	GE Healthcare UK Limited, Little Chalfont, UK

2.6. Primer

All listed primers were supplied by Invitrogen (Regensburg, Germany). The primers were diluted in UltraPure water (Biochrom) to a final concentration of 10 µM.

Table 10: Primers used for amplification of the promoter regions[2]

Gene	Forward (5' → 3')	Reverse (5' → 3')
SEPP	atgctcgagAGATATGGGACCCCAAAAGG	atggatatcAGTCCTGTTGTTTACCTCACC
GPX1	atgggtaccAGAGGAGCCACCAGTTCTCA	atggctagcCCCCGAACAAGCACTGTAAG
GPX2	atgggtaccTTAGCAGATGTCCTGCGATG	atggctagcCCCCACCTGTAAGTGCTGTT
GPX4	atgggtaccAAGAAAACCCTCCAGACTTGTG	atggctagcAGAGCGCTCATTGGTCAGA
DIO1	atgggtaccCCGCCTCTGGACTTCATTTA	atggctagcCTCGGCAAAGCCAGAGTAAG

Table 11: Primers used for qRT-PCR

Gene	Forward (5' → 3')	Reverse (5' → 3')
HPRT	TGACACTGGGAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
18S	TTGACGGAAGGGCACCACCAG	GCACCACCACCCACGGAATCG
CP	CAAAGGAGATTCGGTCGTGT	TGAGGGAAGAGGTTTGCTGT
DDIT3	TGGGGAATGACCACTCTGTT	CTCCTGGAAATGAAGAGGAAGAA
SEPP	TATGATAGATGTGGCCGTCTTG	TGTGATGATGCTCATGATGGTA
DIO1	TTAGTTCCATAGCAGATTTTCTTGTCA	CTGATGTCCATGTTGTTCTTAAAAGC
GPX1	GGGCAAGGTACTACTTATCGAG	TTCAGAATCTCTTCGTTCTTGG
GPX2	AATGTGGCTTCGCTCTGA	GAAGGTGGGCTGGTATCC
GPX4	GCTGTGGAAGTGGATGAAGA	CTAGAAATAGTGGGGCAGGTC

[2] Small letters indicate the restriction sites and capital letters the gene sequence.

Table 12: Primers used for sequencing

Name	Vector system	Primer sequence (5' → 3')
RV3	pGL4.10[Luc2]	CTAGCAAAATAGGCTGTCCC
RV5	pGL4.10[Luc2]	CCGTCTTCGAGTGGGTAGAA

2.7. Cell lines and consumables

Table 13: List of primary cells and cell lines

Name	Origin	Reference	Full growth media
HepG2	Human hepatoma	ATCC® HB-8065	DMEM:F12 containing 10% FBS, 1% Pen/Strep
Hep3B	Human hepatoma	ATCC® HB-8064	DMEM:F12 containing 10% FBS, 1% Pen/Strep
Hepa1-6	Murine hepatoma	ATCC® CRL-1830	DMEM:F12 containing 10% FBS, 1% Pen/Strep
HEK293	Human embryonic kidney	ATCC® CRL-1573	DMEM:F12 containing 10% FBS, 1% Pen/Strep
Primary, murine Hepatocytes	liver from mouse strain C57BL/6	[Lietzow, et al., 2016]	DMEM High Glucose containing 10% FBS, 1% Pen/Strep, 1% Glutamine, 100 nM Na_2SeO_3

Table 14: List of cell culture solutions

Cell culture reagent	Provider
DMEM:F12, HEPES	GIBCO® -Life Technologies GmbH, Darmstadt, Germany
DMEM High Glucose	Biochrom AG, Berlin, Germany
Fetal Bovine Serum (FBS)	GIBCO® -Life Technologies GmbH, Darmstadt, Germany
FuGENE HD transfection reagent	Promega, Mannheim, Germany
Opti-MEM I Reduced Serum Medium	GIBCO® -Life Technologies GmbH, Darmstadt, Germany
Penicillin-Streptomycin (10,000 U/mL)	GIBCO® -Life Technologies GmbH, Darmstadt, Germany
TrypLE™ Express	GIBCO® -Life Technologies GmbH, Darmstadt, Germany
Dulbecco's Phosphate Buffered Saline; DPBS (1x)	GIBCO® -Life Technologies GmbH, Darmstadt, Germany

2.8. Vector systems

Table 15: List of vector systems

Name	Company
pGL4.74[hRluc-tk]	Promega, Mannheim, Germany
pSEAP2-Control	Promega, Mannheim, Germany
pGL4.10[*Luc2*]	Promega, Mannheim, Germany
pGL3-Basic	Promega, Mannheim, Germany
pGL4.26[Luc2/minP/Hygro]	Promega, Mannheim, Germany
pCDH cDNA cloning vector	System Biosciences, Palo Alto, USA

2.9. Laboratory equipment

Table 16: List of laboratory equipment

Equipment	Company
ARPEGE 140, liquid nitrogen tank	Air Liquide- DMC, Paris, France
Autoclave Varioklav	H+P Labortechnik, Oberschleißheim, Germany
Cell culture microscope Wilovert 30 Standard	Helmut Hund GmbH, Wetzlar, Germany
Centrifuge 5415D	Eppendorf, Hamburg, Germany
Centrifuge Megafuge 1.0R	Heareus Sepatec GmbH, Hanau, Germany
Clean bench Model 1.2 (HeraSafe)	Heareus Sepatec GmbH, Hanau, Germany
CO_2-incubator (HeraCell)	Heareus Sepatec GmbH, Hanau, Germany
CO_2-incubator (CB line)	Binder, Tuttlingen, Germany
Electrophoresis chamber (horizontal) Blue Marine 200	Roth, Karlsruhe, Germany
Gel documentation system AlphaImager®EC	Alpha Innotec, San Leandro, Germany
Hemocytometer Neubauer-improved (0.01mm)	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Heating block Thermomixer comfort	Eppendorf, Hamburg, Germany
iCycler™ Thermal Cycler	Bio-Rad Laboratories, Munich, Germany
Laboratory water bath 1083	GFL GmbH, Burgwedel, Germany
Magnetic stirrer R3T	MLW, Würzburg, Germany
Microwave oven Micro	Whirlpool, Schorndorf, Germany
PCR-Cycler	MWG Biotech, Ebersberg, Germany
pH-Meter inoLab Benchtop	WTW, Weilheim, Germany
Pipette 0,5-10uL, 10-100uL, 100-1000uL	Eppendorf, Hamburg, Germany
Pipette filler accu-jet® pro	Brand, Wertheim, Germany
Plate photometer Mithras LB 940	Berthold Laboratories, Bad Wildbad , Germany
Plate photometer Model 3550 Microplate Reader	Bio-Rad Laboratories, Munich, Germany
Plate shaker Titramax 1000	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany
Refrigerated centrifuge 5417R	Eppendorf, Hamburg, Germany
Roller incubator TRM-V	IDL, Nidderau, Germany
scale CP 2201, CP 323S	Sartorius, Göttingen, Germany
Spectrophotometer NanoDrop 1000	PEQLAB Biotechnologie GMBH, Erlangen, Germany
TRANS-BLOT® SD Semi-Dry Transfer Cell	Bio-Rad Laboratories, Munich, Germany
Total-reflection X-ray fluorescence Picofox S2	Bruker Nano GmbH, Berlin, Germany
Ultrapure water unit EASYpure UVTM	Barnstead Int., Dubuque, USA
Ultrasound device Labsonic® M	B. Braun Biotech, Melsungen, Germany
Vortex REAX 2000	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany
X-ray film cassette	Kodak, Rochester, USA

2.10. Software and databases

Table 17: List of software and databases

Software/database	Publisher
BIOGPS	http://biogps.org/#goto=welcome
Bio-Rad iCycler iQ 3.0	Bio-Rad Laboratories, Hercules, USA
BLAST	http://www.ncbi.nlm.nih.gov/BLAST/
BLAT Search	http://genome.ucsc.edu/cgi-bin/hgBlat
Double Digest Finder	http://66.155.211.155/nebecomm/DoubleDigestCalculator.asp
EndNote X8	Thomson Reuters, New York, USA
Genevestigator	https://www.genevestigator.com/gv/
GraphPad Prism 4	GraphPad Software, La Jolla, USA
MatInspector, Genomatrix	http://www.genomatix.de/matinspector.html
Microsoft Office 2013	Microsoft Deutschland, Unterschleißheim, Germany
National Center for Biotechnology (NCBI)	http://www.ncbi.nlm.nih.gov/
OMIM	http://www.ncbi.nlm.nih.gov/omim
Primer 3 Version 4.0	http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
UCSC	http://genome.ucsc.edu/
UniProt	http://www.uniprot.org/

2.11. Cell culture

2.11.1. Cell maintenance

All cell were cultured in full growth medium in a humidified CO_2-incubator at 37°C, 5% CO_2 and 20% O_2. The full growth medium consists of medium (either DMEM: F12 or DMEM High Glucose) supplemented with 10% FBS and 1% Pen/Strep. For serum-starvation and experimental procedures, cells were cultured in starvation medium which consists of serum-free medium (either DMEM:F12 or DMEM High Glucose) supplemented with 1% Pen/Strep.

For strain maintenance, cells were cultured as recommended by the supplier. The cell lines were passaged twice per week in a ratio of 1:4 to 1:6 in a T75 (75 cm^2) cell culture flask. Thereto, complete growth medium was removed and cells were briefly washed with 1x DPBS. 1 mL TrypLE™ Express solution was added to the cells and incubated for five minutes in the CO_2-incubator. Separation of the cells was observed under an inverted microscope. The reaction was stopped by adding 9 mL full growth medium to the cell suspension. The cells were aspirated by gently pipetting. The diluted cell suspension was then transferred in a sub-cultivation ratio of 1:4 to 1:6 to a new cell culture flask and cultured as mentioned above.

2.11.2. Cell counting

The cells were seeded in a defined cell density depending on the experimental set-up. To this end, cells were washed, trypsinised, and aspirated as described before. For cell counting, a subset of cell suspension was mixed in a ratio 1:2 to 1:10 with Trypan Blue solution (Sigma-Aldrich, Hamburg, Germany) in order to visualise vital cells. 10 µL of this mixture were transferred to a hemocytometer and living cells were counted under a light microscope. The average of counted cells was then multiplied by the dilution factor and 10^4 in order to estimate the number of cells per mL.

2.11.3. Freezing and thawing of cells

All cell stocks were stored in 1 mL freezing medium (40% medium, 50 % FBS and 10% DMSO (Sigma-Aldrich, Hamburg Germany)) in liquid nitrogen. In order to thaw cells, cell stocks were taken from liquid nitrogen storage and quickly thawed at 37°C. The cell suspension was then diluted in 15 mL full growth medium and centrifuged at 800 rpm and RT for five minutes. The medium was then removed; cells were re-suspended in 10 mL fresh full growth medium and seeded into a T75-cell culture dish.

In order to freeze cells, confluent grown cells were washed, trypsinised and aspirated as described previously. The cells were then pelleted at 800 rpm and RT for five minutes. After centrifugation, the medium was aspirated and cells were re-suspended in 4 mL freezing medium (50% cell culture medium without additives, 40% FCS and 10% DMSO). 1 mL cryo-vials were prepared, stored overnight at -80°C and finally transferred to liquid nitrogen.

2.11.4. Primary cells

Isolated primary, murine hepatocytes were obtained from a collaboration with the working group of Dr. Renko and Prof. Köhrle (Institute for Experimental Endocrinology, Charité, Berlin, Germany) and isolated as described earlier [Rathmann, et al., 2015]. The hepatocytes were seeded in full growth medium at a density of $0.15x10^6$ cells/$3.5cm^2$ and incubated for three hours under standard conditions. After three hours, the medium was exchanged with fresh full growth medium and cells were further incubated overnight. Prior to each experiment, the medium was exchanged for DMEM 1g/L D-Glucose medium containing 1% Glutamine and 1% Pen/Strep for 24 hours.

2.11.5. Cell viability assay

In order to test the cells viability, an MTT-test assessing cell metabolic activity was performed. The yellow tetrazole MTT, short for 3-(4,5-Dimethylthiazol-2-Y)-2,5-Diphenyltetrazolium Bromide, is reduced to purple formazan in living cells. The formazan accumulates in the form of cell aggregates and correlates to the cell viability. Briefly, 20,000 cells/well were seeded in a 96-well plate. After 24 hours, the full growth medium was removed and cells were incubated for further 24 hours in starvation medium. Cells were then stimulated with different concentrations of pro-inflammatory cytokines, aminoglycosides and Se. After 48 hours, the medium was removed, cells were washed once in 1x PBS and 225 µL fresh starvation medium and 25 µL 12 mM MTT (MTT in ddH_2O) was added per well. The cells were incubated for one to three hours in a CO_2-incubator at 37°C. The reaction was stopped by removing the MTT-containing medium. The cells were lysed for ten minutes using 100 µL lysis buffer (1:$3.3x10^{-3}$ 37% HCL in isopropanol). Finally, the absorbance of the accumulated formazan was measured at 492 nm in a micro plate reader. The cell viability was calculated as difference in absorption (ΔE) of stimulated and unstimulated cells.

2.12. Cloning

2.12.1. Cloning of promoter regions

Primer design

In order to investigate the regulation of selenoproteins by pro-inflammatory cytokines, aminoglycosides and Se, an approximately 1000 bp long promoter region (directly upstream of the transcription start of a set of selenoprotein genes) was amplified from human genomic DNA. Cloning primers were designed using Primer3. For further cloning procedures, restriction sites were added to the 5' end of the primer sequence (Table 10). Genomic sequences were obtained from NCBI.

Amplification of promoter regions

A gradient-PCR was performed for each primer pair in order to determine the optimum annealing temperature. For the amplification of the promoter regions, the KAPA HiFi HotStart ReadyMix PCR Kit was used as followed:

34.5-x µL	UltraPure water (Biochrom)
10 µL	5x KAPA HiFi Buffer
1.5 µL	10 mM KAPA dNTP Mix
1.5 µL	10 µM forward primer (Invitrogen)
1.5 µL	10 µM reverse primer (Invitrogen)
x µL	50 ng DNA template
1 µL	1 U/µL KAPA HiFi HotStart DNA Polymerase
50 µL	final volume

The gradient-PCR was then performed under the conditions given in Table 18.

Table 18: Gradient-PCR cycle condition

No.	Temperature [°C]	Duration [min]	Step	Remarks
1	95	3:00	initial denaturation	Repeat step 2 to 4 for 35 cycles
2	98	0:20	denaturation	
3	60 ± 10	0:15	primer annealing	
4	72	0:30/kb	elongation	
5	72	1 min/kb	final elongation	

The expected PCR product size was verified via agarose gel electrophoresis. The promoter region was finally amplified with optimised annealing temperature under similar conditions.

Purification of PCR-product

The amplified promoter fragment was purified using the Perfectprep® Gel Cleanup Kit (Eppendorf) and the according protocol. For the detailed protocol, please refer to the Perfectprep® Gel Cleanup manual. The DNA concentration of the purified PCR-product was measured using a UV/VIS-spectrophotometer.

Restriction digestion

In order to clone the amplified promoter fragments into the vector pGL4.10[Luc], 500 ng purified PCR-product and 1-5 µg of the target vector were digested with suitable restriction enzymes. Thereto, 1x NEBuffer, 1x BSA (New England Biolabs GmbH), 0.25 µL (20,000 U/mL) restriction enzyme (New England Biolabs GmbH) and x µL UltraPure water (Biochrom) (10 µL final volume) were added to the PCR-product or target vector, and incubated for three hours at 37°C. The digested PCR-product was purified using the Perfectprep® Gel Cleanup Kit (Eppendorf).

Vector dephosphorylation

To avoid self-ligation of the vector, the prior digested vector was dephosphorylated using Shrimp Alkaline Phosphatase (Fischer Scientific, Schwerte, Germany). 1-5 µg vector and 1 µL SAP (1 U/µL) were diluted in 1x SAP reaction buffer to a final volume of 20 µL and incubated for 15 minutes at 37°C. The reaction was incubated for 30 minutes at 65°C in order to heat inactivate the SAP. The dephosphorylated vector was finally purified with Perfectprep® Gel Cleanup Kit (Eppendorf).

Ligation

The target vector and the PCR-fragment were mixed in a molar ratio of 1:3. 1 µL T4 DNA Ligase, 2x T4 Ligase Buffer (T4 DNA Ligase Kit, New England Biolabs GmbH) and x µL of UltraPure water (Biochrom) were added to a final volume of 20 µL. The ligation reaction was then performed at 16°C overnight.

Transformation of *E.coli* cells DH5α

For cloning purposes, the non-pathogenic *E.coli* strain *E.coli* (DH5α) was used. Due to multiple mutations it allows a high-efficiency transformation (genotype: dlacZ Delta M15 Delta (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-mK+) supE44 thi-1 gyrA96 relA1). Competent *E.coli* DH5α cells were pre-chilled on ice and ligated pGL4.10[promoter-Luc] vector was added to competent cells (ratio v/v 1:10). After 20 minutes on ice, cells were heat-shocked for 30-45 s at 42°C and immediately cooled down on ice. Cells were then incubated for one hour at 37°C in 500 µL ampicillin-free LB medium. Depending on the cell density, 10-200 µL of cell suspension was plated onto ampicillin-containing (100 µg/mL) LB agar plates and incubated over night at 37°C.

Mini preparation

Single grown colonies were picked from overnight grown, transformed *E.coli* DH5α cells. From these colonies, plasmid-DNA was isolated using the Fast Plasmid Mini Kit (Table 6). The concentration of plasmid DNA was measured using spectrophotometer.

Test restriction digestion

In order to verify whether the isolated plasmids contain the desired promoter fragment, a restriction digestion test was performed. The correct fragment size was confirmed via agarose gel electrophoresis.

Sequencing

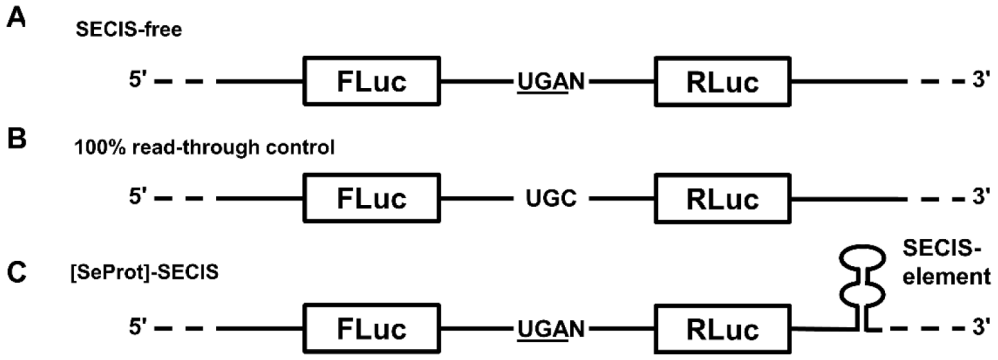
Positive identified clones were sent for sequencing to LGC genomics (Berlin, Germany). To this end, 1 µg DNA were mixed with 4 µL of 5 µM sequencing primer (Table 12) to a final volume of 14 µL in UltraPure water (Biochrom). Sequences were then compared to reference sequences using ClustalW2 Multiple Sequence Alignment (EMBL-EBI). The reference sequences are based on NCBI database.

Plasmid purification

In order to purify the plasmid DNA, positive *E.coli* DH5α clones were cultivated overnight in 50 mL ampicillin-containing LB-medium. The cells were cultivated until an OD_{600} of 1.0-1.5 was achieved and then sequentially centrifuged for ten minutes at RT and 5,000 x *g*. The plasmid DNA was isolated and purified using PureYield™ Plasmid Maxiprep Kit (Table 6) and according to the manual of the supplier.

2.12.2. Cloning of Se-dependent reporter constructs

For the design of all Se-dependent reporter constructs, a fusion protein of full-length Firefly luciferase (FLuc) and Renilla luciferase (RLuc) was cloned into the multiple cloning site of pCDH cDNA cloning vector (Table 15) as described in section 2.12.1. The fusion protein reading-frame was interrupted by an in-frame UGA codon, coding for both the Opal-stop codon and Sec-insertion. The 3'UTR contained the selenoprotein-specific SECIS-elements of GPX1, GPX4 or SEPP, or a SECIS-free sequence (negative reporter). Fragment sizes and primers of the specific SECIS-elements are given in Figure 10.



SECIS-element	length [nt]	5' site	3' site
GPX1	137	CGGCTGCTTGGCAGTTGC	GGATCAACAGGACCAGCA
GPX4	119	CCTGCCCACGCCCTTGGA	GCAGGCCATGGGACCTTC
SEPP (SECIS1)	142	AGGTGATTGCAGCTTTTG	TTGTTGTTCTTCCTCCAT
SEPP (SECIS1&2)	571	AGGTGATTGCAGCTTTTG	ATTCTTGCTTAATAGTATTAAC

Figure 10: Se-dependent reporter constructs.

The Se-dependent reporters constructs were designed as fusion protein of full-length Firefly luciferase (FLuc) and Renilla luciferase (RLuc) interrupted by an in-frame (A) UGA codon (SECIS-free control), (B) UGC codon (100% readthrough control) or (C) UGA codon with additional selenoprotein-specific SECIS-elements of *GPX1, GPX4* or *SEPP* in the 3'-untranslated region [SeProt]-SECIS.

A positive reporter was constructed by the replacement of the UGA triplet for a UGC triplet coding for cysteine allowing a stimulation independent readthrough (100% readthrough control).

2.13. Reporter gene assays

2.13.1. Reporter gene assays in transiently transfected cell lines

For a transient transfection, 0.095 µg plasmid and 0.005 µg control plasmid (pSEAP2-Control) were dissolved in Opti-MEM cell culture media. 2.5x FuGENE transfection reagent was added and incubated for 15 minutes at RT. $2x10^4$ cells per well were added to the reaction mixture, carefully mixed and seeded in a 96-well plate at a total volume of 100 µL. Next day, cells were starved for one hour in starvation media and stimulated for a further 17 hours with standard concentrations of IL-6, TNFα or IL-1β dissolved in starvation media.

For normalisation reasons, 50 µL supernatant was transferred to a new 96-well cell culture plate and incubated for 15 minutes with 20 µL SEAP reagent (ratio v/v 1:10 CSPD® substrate with Sapphire-II™ Enhancer (Applied Biosystems, Foster City, USA) and SEAP buffer (1 M EDTA, 0.5 mM $MgCL_2$)). The SEAP activity was measured as relative light units (RLU) using microplate luminometer. In parallel, the cells were washed once with 1x PBS and lysed for 20 minutes at RT using lysis buffer (1:5 PassiveLysis Buffer diluted in ddH_2O). The activity of Firefly luciferase (FLuc) was measured by adding 20 µL Luciferase substrate (Dual-Luciferase® Reporter Assay System 10-Pack; Promega, Mannheim, Germany). The FLuc activity is also given as RLU.

2.13.2. Reporter gene assays in stable transfected HEK293 cells

For stable transfection of HEK293 cells, $0.5x10^5$ cells/well were seeded into six-well cell culture dishes and incubated overnight at 37°C, 5% CO_2 and 20% O_2. The medium was changed after 24 hours and transfected as follows. 2 µg plasmid and 6 µl of FuGENE transfection reagent were diluted in 100 µL Opti-MEM cell culture medium and incubated for 15 minutes at RT. The transfection mixture was then added to each well. The medium was exchanged with full growth medium after 24 hours. Subsequently, growth medium was again changed after 24 hours to medium containing 5 µg/mL puromycine (ThermoFisher) for positive selection.

Stably transfected HEK293 cells were seeded in pre-coated (1:40 poly-L-lysine diluted in 1x PBS) 48-well cell culture dishes at 50,000 cells/well. After an incubation for 24 hours in

starvation medium, cells were stimulated with either AG or Se dissolved in starvation medium. The medium was removed after 48 hours and cells were lysed in 80 µL lysis buffer (1:5 PassiveLysis Buffer diluted in ddH_2O) for ten minutes at RT. 20 µL cell lysate was transferred to a white 96-well cell culture plate and RLuc and FLuc activities were determined independently using Renilla-Juice detection system or Beetle-Juice detection system (

Table 6). RLuc and FLuc activities were determined as RLU using a microplate luminometer.

2.14. Analysis of transcript level

Isolation of mRNA

$0.5x10^6$ cells were seeded to six-well cell culture dishes in full growth medium. The next day, cells were starved for 24 hours in starving media and stimulated for a further 48 hours with the IL-6, G418, geneticin or Na_2SeO_3, diluted in starvation medium.

The cells were washed with 1x PBS and 1 mL peqGOLD TriFAST (PEQLAB Biotechnologie GmbH, Erlangen, Germany) was added to each well. After ten minutes of incubation at RT, the cell suspensions were transferred to tubes (Eppendorf) and lysed in a tissue lyser (5 min at 15 s^{-1}). For phase separation, 200 µL Chloroform was added to each sample and cell lysate was centrifuged for ten minutes at RT and 14,000 rpm. The aqueous phase was transferred to 500 µL ice-cold isopropanol and precipitated at -20°C overnight. The reaction mixture was again centrifuged for ten minutes at 4°C and 14,000 rpm in order to precipitate the RNA. The supernatant was removed and the cell pellet was washed twice with 75% DEPC-EtOH (EtOH absolute in DEPC-H_2O). Finally, the supernatant was removed and the pellet was dried for ten minutes at RT. The dried cell pellet was then dissolved in 30 µL DEPC-H_2O (0.1 mL DEPC/100 mL H_2O) and RNA concentration was measured using a spectrophotometer.

cDNA synthesis

1 µg RNA was translated into cDNA using iScript cDNA Synthesis Kit (Bio-Rad). According to the manufacturer's protocol, the following reaction mixture was used for one reaction:

4.0 µL	5x iScript reaction mix
1.0 µL	iScript reverse transcriptase
15-x µL	nuclease-free water
x µL	0.5 µg RNA template
20 µL	final volume

For cDNA synthesis, the cycler conditions given in Table 19 were used.

Table 19: Cycle conditions for the cDNA synthesis

Cycle	Temperature [°C]	Duration [min]	Step
1	25	5:00	annealing
2	42	30:00	elongation
3	85	5:00	reaction inactivation
4	8	-	storage

Quantitative RT-PCR

The mRNA expression level in HepG2 cell was determined using qRT-PCR. For one qRT-PCR reaction, the following reaction mixture was used:

5.0 µL	Sybr Green Dye (Life Technologies)
4.5 µL	Aqua Pure (Biochrom)
0.25 µL	10 µM Reverse Primer
0.25 µL	10 µM Forward Primer
5.0 µL	cDNA template
15 µL	final volume

The reaction mixture was transferred to a 96-well plate and qRT-PCR was performed under the cycle conditions given in Table 20.

Table 20: Cycle conditions for the qRT-PCR

No.	Temperature [°C]	Duration [min]	Step
1	95	15:00	initial denaturation
2	95	0:15	denaturation
3	58	0:30	annealing
4	72	0:30	elongation
melting curve			
5	50-90	0:15	denaturation

For normalisation reasons, different housekeeping genes were tested for their stability to pro-inflammatory cytokine, aminoglycoside and Se-stimulation. Subsequently, the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) was chosen for stimulation with IL-6 ± Se and 18S RNA for stimulations with aminoglycosides. The transcript regulation was then analysed via the $2^{(-\Delta\Delta t)}$ method.

2.15. Protein chemical methods

2.15.1. Isolation of proteins

For protein isolation, $0.5x10^6$ HepG2 or Hepa1-6, and $0.25x10^6$ Hep3B cells or primary hepatocytes were seeded into six-well cell culture dishes. After 24 hours, cells were starved for another 24 hours and then stimulated with pro-inflammatory cytokines, AG and Se, diluted in the according starvation medium. The conditioned medium was removed after 48 hours and stored at -20°C until further use. The cells were washed with 1x PBS, 250 µL homogenisation buffer (Table 3) was added and cells were collected using a cell scraper. In order to homogenise the cells, the cell suspension was treated with ultrasound (cycle 0.6; amplitude 100%) eight times. The samples were stored at -20°C until further use.

2.15.2. BCA-Assay

The protein concentrations of the cell lysates were measured using Pierce BCA Protein Assay Kit (

Table 6). Following the manufacture's protocol, 25 µL standard (BSA standard), and 25 µL sample (1:2.5 diluted in homogenisation buffer) were transferred to a 96-well plate. The measurement was performed in duplicates. Next, 200 µL of working reagent (BCA Reagent 1

and BCA reagent 2; ratio v/v 50:1) was added, thoughtfully mixed on a plate shaker and incubated for 30 minutes at 37°C. The absorbance was measured at 592 nm in a microplate reader. The protein concentration was finally determined according to a standard curve resulting from internal BCA standards with known protein concentrations.

2.15.3. Western blot

Protein lysates were adjusted to the same protein concentration using homogenisation buffer and 4x sample loading buffer (Table 4). The adjusted samples were incubated for five minutes at 95°C. For an optimal protein size separation, a discontinuous electrophoresis with differently concentrated separation gels depending on the protein size were used (Table 21).

Table 21: Composition of stacking and separation gel

Component	5% Stacking gel (4 mL)	10% Separation gel (10 mL)	12.5% Separation gel (10 mL)
ddH_2O	2 mL	2.5 mL	1.25 mL
20% Gel solution	1 mL	5 mL	6.25 mL
Separation gel buffer (pH 8.8)	-	2.5 mL	2.5 mL
Stacking gel buffer (pH 6.8)	1 mL	-	-
10% APS	40 µL	100 µL	100 µL
TEMED	4 µL	10 µL	10 µL

20-50 µg protein and 5 µL TriColor protein ladder (3.5-245 kDa; biotechrabbit GmbH, Henningsdorf, Germany) were loaded onto the gel. In parallel, 50 µL (final volume) of conditioned medium was used and respectively diluted in 4x sample loading buffer. The separation was performed at 80 V (stacking gel) and 120 V (separation gel) until the desired separation of protein samples was accomplished.

Size fractioned samples were transferred onto nitrocellulose membrane (Optitran BA-S85, GE Healthcare) using 1x transfer buffer (Table 4) for semi-dry sandwich principle. Depending on the protein size, the blotting was performed at 25 V for 30-45 minutes. In order to guarantee a successful protein transfer, the nitrocellulose membrane was stained with 1x Ponceau S staining solution (Table 4). The staining solution was removed and the membrane was blocked for one hour in blocking solution (Table 4) at RT.

The membrane was then incubated with the primary antibody (diluted in blocking solution) overnight at 4°C. The next day, the nitrocellulose membrane was washed three times with 1x TBS-Tween solution and incubated with the secondary antibody (diluted in blocking solution) for one hour at RT. The nitrocellulose membrane was again washed three times with 1x TBS-Tween, incubated for one minute in ECL™ Prime (GE Healthcare) solution and exposed to an X-ray film (Amersham Hyperfilm™ ECL, GE Healthcare) for one minute. The X-ray film was developed using the developer (GPX Developer and replenisher, Kodak), followed by fixer (GPX Fixer and replenisher, Kodak). For normalisation reasons, all performed Western blot were normalised to either Ponceau S staining or the housekeeping gene β-actin.

2.15.4. Dot blot

The Dot blot technique allows a semi-quantitative detection of proteins without a prior size-fractioning. A nitrocellulose membrane was thereto shortly equilibrated in 1x transfer buffer and mounted into the Dot blot apparatus. The membrane was washed twice with 200 µL 1x TBS (Table 4) and pre-loaded with 200 µL 1x TBS/well. Depending on the used cell line, 150-250 µL of conditioned media was directly transferred to each well. The vacuum was applied and the membrane afterwards washed twice with 200 µL 1x TBS. In order to guarantee successful and equal protein transfer, the nitrocellulose membrane was stained with 1x Ponceau S staining solution. The further procedure is in parallel to Western blot technique.

2.15.5. Selenoprotein P-ELISA

In order to quantify SEPP concentrations in conditioned cell culture media; the colorimetric enzyme immunoassay Selenotest ELISA (ICI-immunochemical intelligence GmbH, Berlin, Germany) was used. To this end, 100 µL of pre-diluted conditioned media (1:4 in sample dilution buffer (BSD)), internal standards, calibrators and blank were transferred to a 96-well plate. The 96-well plate is pre-coated with SEPP capture antibody. The samples were incubated for one hour at RT. After washing four times with 250 µL washing buffer (1x BWA in ddH_2O), samples were incubated for one further hour at RT with 100 µL biotin-labelled SEPP-detection antibody (1:110 DAB in antibody dilution buffer (BDA)). After additional washing steps, the samples were incubated with 100 µL streptavidin-peroxidase-conjugate solution (1: 110 POD in POD dilution buffer (BPO)) for one hour at RT. The samples were again washed and incubated with 100 µL peroxidase substrate solution (TMB) for one further hour at RT. The reaction was then stopped by adding 100 µL stop solution (STO) and absorbance was measured at 450 nm in a microplate reader. The SEPP protein concentrations were measured in duplicates and calculated according to an internal standard curve.

2.16. Enzyme assays

2.16.1. GPX enzyme activity assay

In order to determine the GPX enzyme activity, the cells were lysed as described previously. 5-25 µL of cell homogenates were transferred to a 96-well plate. As controls, 5 µL human serum (positive control), Se-free treated cell homogenate (negative control) and blank (background control) were used in every experimental set-up. All samples were measured in duplicates. The following reaction mixture was prepared for one 96-well plate, and 225 µL of this mixture was used per reaction.

21.5 mL	Basic buffer (Table 5)
250 µL	10% Triton-X (1:10 Triton-X in ddH_2O)
150 µL	53.7 mM NADPH (NADPH in 10mM KPO_4-buffer pH 7.0)
250 µL	100 mM reduced GSH (GSH in ddH_2O)
25 µL	Glutathione reductase (GR, 1:7 in 3.2 M ammonium sulphate)
22.2 mL	final volume

To start the reaction, 10 µL $3.75x10^{-3}$% H_2O_2 (1:8000 30% H_2O_2 in ddH_2O) was added and GPX enzyme activity was measured as decreasing absorbance (ΔE) at 340 nm using a spectrophotometer. GPX enzyme activity is thereby measured indirectly as follows:

$$ROOH + 2\,GSH \xrightarrow{GPX} ROH + GSSG + H_2O$$
$$NADPH + H^+_{(340nm)} + GSSG \xrightarrow{GSR} NADP^+ + 2\,GSH$$

The GPX enzyme activity was determined as ΔE per minute, normalised to protein concentration and calculated as follows

$$relative\ GPX\ activity\ \left[\frac{nmol}{min \times mg}\right] = \frac{\frac{\Delta E_{340nm}}{min} \times volume\ [L]}{6200\ \left[\frac{1}{M \times cm}\right] \times d[cm] \times m_{protein}[mg]}$$

With m= protein amount [mg] and $6200\ \left[\frac{1}{M \times cm}\right] \times d[cm]$ as light path.

2.16.2. TXNRD enzyme activity assay

The cell lysates were homogenised as described previously and 10-25 µL of these homogenates were transferred to a 96-well plate. The samples and controls were measured in duplicates. Se-treated cells (positive control), Se-free treated cells homogenate (negative control) and blank (background control) were used as control. 200 µL of the following reaction mixture was added to every sample.

158.2 µL	ddH_2O
20 µL	1M potassium phosphate buffer (pH 7.0)
16 µL	63.07 mM DTNB (DTNB in DMSO)
4 µL	0.5 M EDTA (EDTA in ddH_2O pH 8.0
1 µL	53.7 mM NADPH (NADPH in 10mM KPO_4-buffer pH 7.0)
0.8 µL	50 g/L BSA (BSA in 10 mM 10mM KPO_4-buffer pH 7.0)
200 µL	final volume

The increasing absorbance (ΔE) was measured at 412 nm using a spectrophotometer. The TXNRD enzyme activity is indirectly determined as follows:

$$DTNB \xrightarrow{R-SH} 2\ Nitro\ 5\ mercapto\ benzoic\ acid_{(412nm)} + RS - SR$$

$$NADPH\ H^+ + TXNRD \xrightarrow{RS-SR} TXNRD + NADP + R(SH)_2$$

The resulting ΔE was then normalised to the protein concentration and the activity was calculated as follows:

$$TXNRD\ activity\ \left[\frac{nmol}{min \times mg}\right] = \frac{\frac{\Delta E_{412nm}}{min} \times volume\ [L]}{2 \times 13000\ \left[\frac{1}{M \times cm}\right] \times d[cm] \times m_{protein}[mg]}$$

With m= protein amount [mg], $13000\ \left[\frac{1}{M \times cm}\right] \times d[cm]$ as extinction coefficient and light path, respectively, and 2 as stoichiometric ratio to consider the conversion of DTNB to two 2-Nitro-5-mercapto-benzoid-acid (TNB).

2.16.3. DIO1 enzyme activity assay

For the DIO1 activity enzyme assay, the cells lysates were processed as indicated previously. 40 µL of the cell lysate was hence transferred to 8-tube PCR-stripes and 10 µL homogenisation buffer or 10 µL PTU (10 mM) as background control, was added. The reaction was started by adding 50 µL of the following substrate mix:

10 µL	1 M potassium phosphate buffer (pH 6.8)
4 µL	DTT (1 M)
1 µL	1 mM rT3 (Sigma-Aldrich, Hamburg, Germany)
35 µL	ddH_2O
50 µL	final volume

The samples and controls were measured in duplicates. For each experiment, Se-free (negative control) and 100 nM Na_2SeO_3 (positive control) supplemented cells were used. The reaction was incubated for six hours at 37°C in a thermo shaker and immediately stopped on dry ice.

In order to precipitate the cellular debris, the reaction was centrifuged for five minutes at 12,000 rpm and 4°C. 75 µL of the supernatant and 100 µL of 10% acetic acid were transferred to a pre-equilibrated Dowex column (Dowex® 50W X8, Serva, Heidelberg, Germany), and vacuum was applied. The discharged supernatant was collected and diluted 1:8 with 10% acetic acid to a final volume of 50 µL. The DIO1 enzyme activity was measured as amount of free iodide, released during DIO1-catalysed degradation of rT_3 to 3,3'-T_2.

The released iodide was determined in the Sandell-Kolthoff reaction as follows:

$$2\ Ce\ (IV)_{(415nm)} + As\ (III) \xrightarrow{iodide} 2\ Ce\ (III) + As\ (V)$$

Thereto, 50 µL of Iodine-determination buffer 1 and buffer 2 were added to 50 µL reaction mixture. The iodide concentration was determined measuring the decreasing rate of absorbance (415 nm) over 21 minutes and converted using as external standard curve. The DIO1 enzyme activity was normalised to the protein concentration and is given in $\frac{pmol}{min \times mg}$.

2.17. Spectrometric determination of Selenium

1 mL of conditioned medium was transferred to a nitrocellulose membrane using Dot blot principle. Defined punches of 4 mm diameter were taken of each dot and bound proteins were lysed by applying 20 µL of 60% HNO_3 (containing a Ga-Standard of 1000 µg/L) to the punch. The reaction mixture was incubated at 70°C for 30 minutes in a thermocycler.

The punch lysates were applied onto quartz glass sample carriers (Bruker, Berlin, Germany). The samples were measured in duplicates using total reflection X-ray fluorescence (TXRF) spectroscope S2 Picofox (Bruker, Berlin, Germany). For the verification of Se-levels, a human serum standard (Seronorm) was used in each experimental set-up.

2.18. Statistics

The Statistical analyses were performed using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, USA). All results are presented as mean ± SEM and the number of replicates is given in the figure legends. If data followed a normal distribution, differences between two groups were tested using student's t-test, multiple-group comparisons were made using one-way ANOVA followed by Dunnett's Posthoc test. Non-normally distributed data were tested using Mann-Whitney-U test or Kruskal-Wallis test for two group respectively multiple group comparisons. The significance is assigned if $P < 0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

3. Results

3.1. Regulation of hepatic selenoprotein metabolism by IL-6

3.1.1. Effects of IL-6 on hepatic SEPP expression

The liver is one of the main effector organs of IL-6 during the immune response. It promotes, i.a. the release of the acute phase protein ceruloplasmin (CP). In parallel, the liver is the central organ of the selenoprotein metabolism, producing several antioxidative acting selenoproteins, e.g. GPX, TXNRD and the Se-transporter SEPP. As SEPP is not only the most important Se-source for peripheral organs but is also negatively affected during acute phase response, hypoxia and IL-6, the question emerged as to how IL-6 effects the expression of SEPP and other selenoprotein in the liver [Becker, et al., 2014; Renko, et al., 2009; Wiehe, et al., 2016].

Using the human hepatoma cell lines HepG2 and Hep3B, the effect of IL-6 on the SEPP expression was investigated. We found that IL-6 strongly down-regulated the SEPP expression level in both cell lines (Figure 11). The IL-6-mediated decrease of SEPP expression effects both extra- and intracellular SEPP (Figure 11 A+B). Furthermore, down-regulation of SEPP protein expression in response to IL-6 occurred in a dose-dependent manner by more than 50% as compared to untreated cells (Figure 11 C). These effects were not linked to toxicity, as a cell viability test of HepG2 cells showed that IL-6 at a concentration range of 0.1-500 ng/mL had no ill effects. Similar to the finding in HepG2 cells, IL-6 down-regulated SEPP expression in Hep3B cells (Figure 11 D).

In order to understand the underlying mechanism of IL-6-mediated SEPP down-regulation, the effect of IL-6 on the *SEPP* transcript level was investigated in HepG2 cells. As *CP* is a target gene of IL-6, it was used as positive IL-6 induction control. IL-6 induced the *CP* transcript level independent of Se-supplementation (Figure 11 E). The *SEPP* transcript level was strongly promoted by Se-supplementation when compared to Se-depleted cells. Although the *SEPP* transcript level was significantly reduced by IL-6 in both Se-supplement and Se-depleted cells, the absolute *SEPP* transcript level in Se-supplemented cells was markedly higher. Overall, these findings indicate that supplemental Se attenuates the inhibitory effect of IL-6.

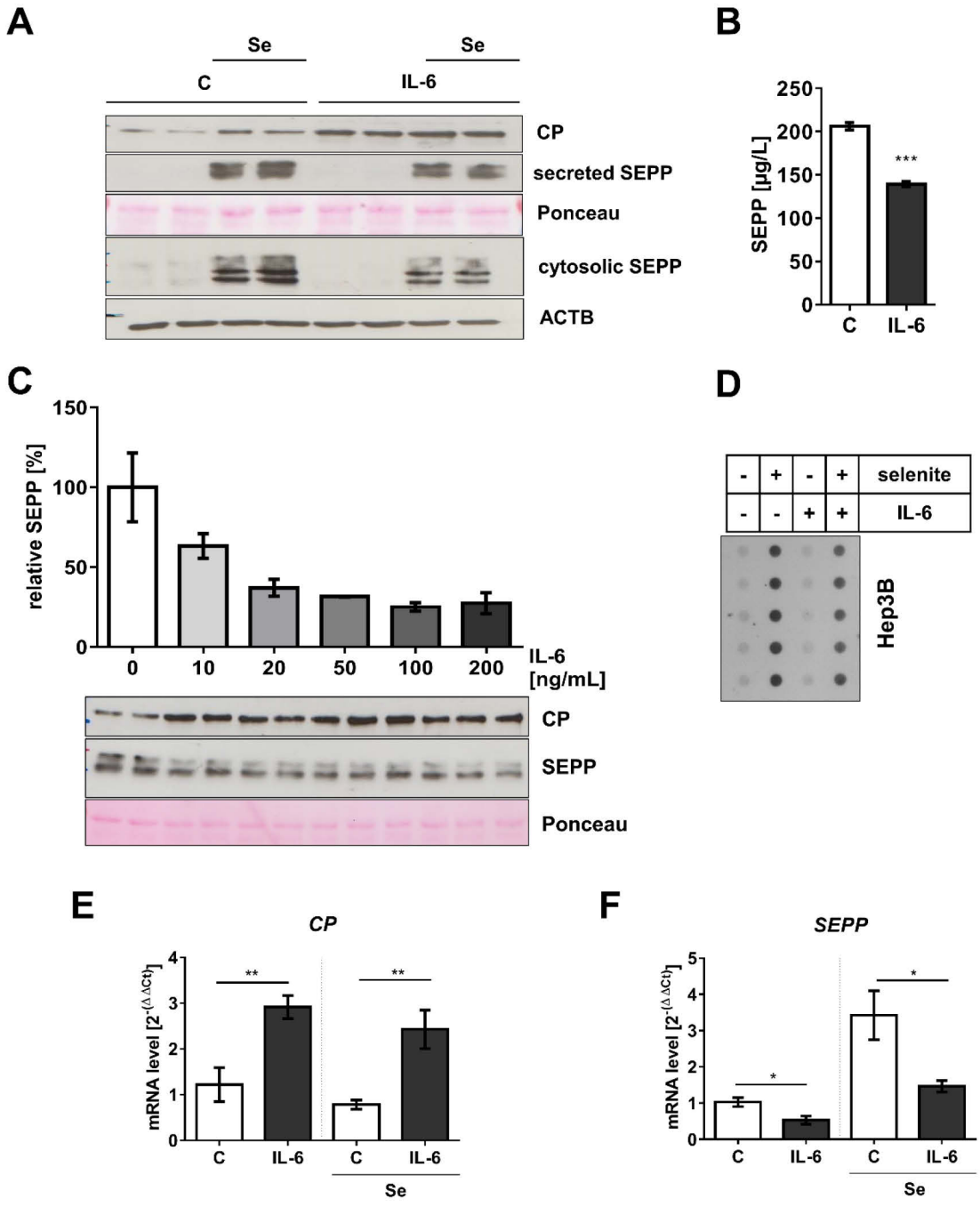


Figure 11: IL-6 down-regulates the SEPP expression in HepG2 and Hep3B cells.

Se-supplemented and Se-depleted HepG2 cells were stimulated with a standard IL-6 concentration of 100 ng/mL for 48 hours. (A) IL-6 markedly induced the expression of its target gene CP and significantly down-regulated the SEPP expression (B) The absolute SEPP-concentration secreted into cell culture medium was significantly reduced by IL-6. (C) IL-6 decreased the SEPP expression by more than 50% in a dose-dependent manner. (D) In parallel, IL-6 reduced the SEPP expression in Hep3B cells as observed by Dot blot analysis. (E) On transcriptional level, IL-6 significantly induced *CP*, while the *SEPP* transcript level was strongly reduced by more than 50% when compared to the control, in both Se-depleted and Se-supplemented cells (Mean ± SEM, n=6).

The regulation of IL-6 target genes is mainly mediated by the activation of the JAK-STAT signalling pathway [Schmidt-Arras and Rose-John, 2016]. As IL-6 signalling is not exclusively mediated via the transcription factor STAT3, it was tested whether STAT3 is the main mediator of IL-6 signalling in HepG2 and Hep3B cells. To this end, HepG2 cells were transiently transfected with different reporters containing the response elements for NF-κB, AP-1 and STAT3. The cells were then stimulated with 0, 1, 10 and 100 ng/mL IL-6 for 24 hours. While no changes of the reporter response containing the NF-κB or AP-1 response elements were observed (Figure 12 A), IL-6 significantly induced the STAT3 response element in a dose-dependent manner (Figure 12 A + B).

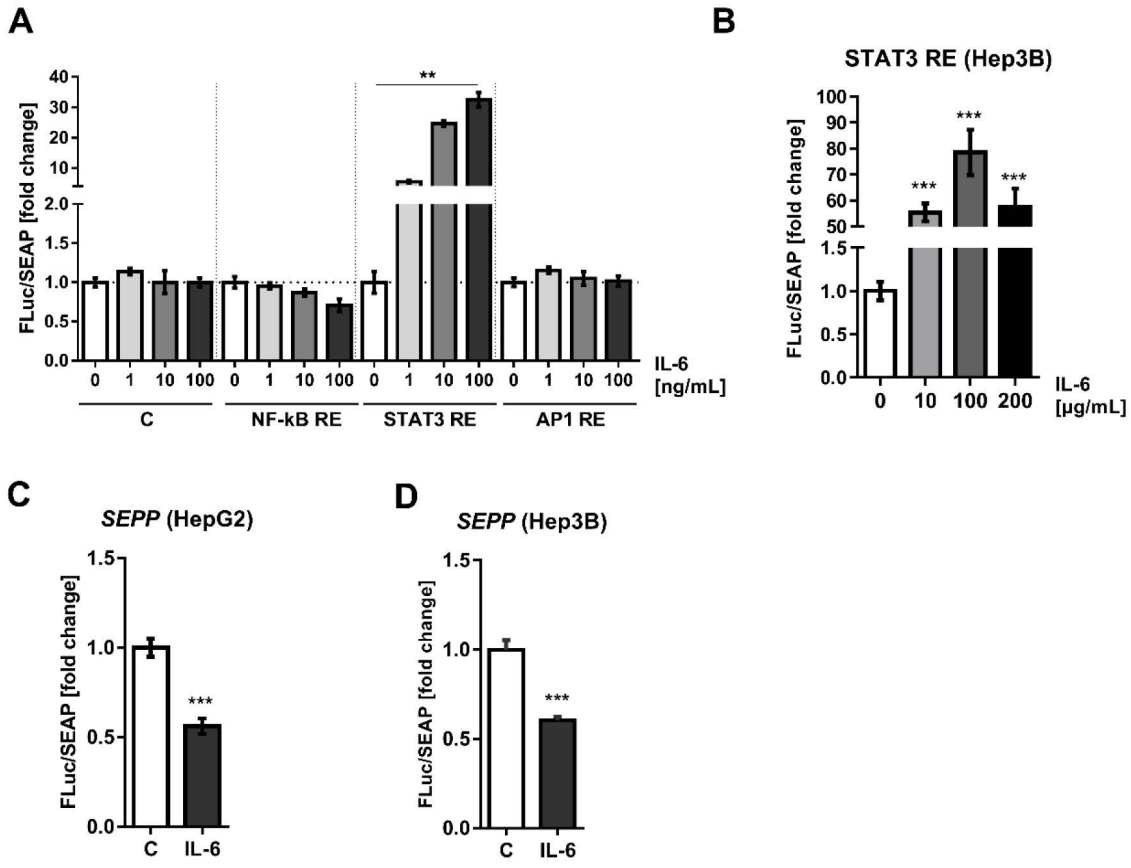


Figure 12: IL-6 down-regulates the *SEPP* promoter activity potentially via STAT3.

HepG2 and Hep3B cells were transiently transfected with reporter construct containing the response element for STAT3, NF-κB or the AP-1 and stimulated with 0, 1, 10 or 100 ng/mL IL-6 for 24 hours. (A) The STAT3 response element, but neither the NF-κB nor the AP-1 response element, were significantly induced by IL-6 in a dose-dependent-manner and in both HepG2 and (B) Hep3B cells. In comparison, IL-6 significantly down-regulated the *SEPP* promoter activity in (C) HepG2 and (D) Hep3B cells (Mean ± SEM, n=4-6).

To test whether IL-6 directly regulates the SEPP expression via the promoter, an approximately 1000 bp long *SEPP* promoter fragment was cloned into a FLuc-containing reporter plasmid. The reporter construct was then transiently transfected into HepG2 and Hep3B cells and subsequently stimulated for 24 hours with 100 ng/mL IL-6. The results show that the *SEPP* promoter activity was significantly down-regulated by IL-6 in HepG2 (Figure 12 C) and Hep3B cells (Figure 12 D) to on average of 50% as compared to the unstimulated control.

Following steps took sets of shorter *SEPP* promoter fragments (874 bp, 599 bp, 298 bp, 224 bp, 161 bp and 93 bp long promoter fragments) generated and cloned into a FLuc-reporter in order to narrow down the promoter region potentially underlying IL-6 signalling. The different *SEPP*-derived reporter constructs were transiently transfected into HepG2 cells and stimulated with increasing IL-6 concentrations for 24 hours. Changes in the promoter activity of the different fragments were analysed in comparison to the full-length *SEPP* promoter (1000 bp). Systemic reduction of the promoter length from 874 bp, to 599 bp, to 298 bp (Figure 13 A) and then to 224 bp, to 161 bp or 93 bp (Figure 13 B) from the transcription start, did not abolish an IL-6-mediated decrease of the *SEPP* promoter activity. Reducing the promoter length to 93 bp, the decrease in the promoter activity remained at > 50% as compared to the full-length control. These findings suggest that IL-6 represses the SEPP expression by targeting the *SEPP* promoter within this 93 bp promoter region.

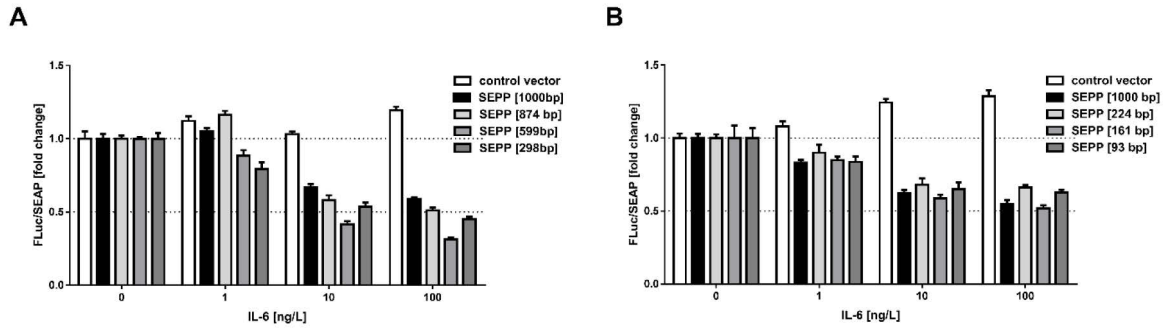


Figure 13: Refining the *SEPP* promoter region underlying IL-6 signalling.

In order to narrow down the promoter region that is responsive to IL-6, the *SEPP* promoter was systemically shortened from (B) 874 bp, to 599 bp, to 296 bp and then (C) to 224 bp, to 161 bp and finally to 93 bp upstream of the transcription start. In all these different promoter constructs, no effect on promoter activity compared to the full-length *SEPP* promoter (1000 bp) was observed. The vector backbone was used as negative control (Mean ± SEM, n=8).

3.1.2. Effects of IL-6 on hepatic GPX, TXNRD and DIO

To overcome the pathogen triggered side of infection, the immune system responds with an increased production of reactive oxygen species (ROS). Uncontrolled increases of ROS may lead to an imbalance in redox homeostasis, which can result in damage to cellular structures and tissues. This ROS imbalance is restored by cellular antioxidative molecules or enzymes such as superoxide dismutase, catalase as well as members of the Se-families namely GPX and TXNRD. As IL-6 decreases the expression of SEPP to ~ 50%, the question arose whether this down-regulation results in a redistribution of limited Se towards the more essential and important antioxidative selenoproteins. To this end, it was tested whether IL-6 may indirectly or directly regulate the expression of these antioxidative proteins.

A potential impact of IL-6 on GPX protein expression levels was evaluated via Western blot analyses and enzyme activity assays (Figure 14). Using the GPX substrate hydrogen peroxide, the absolute GPX enzyme activity in response to IL-6 was studied in Se-supplemented and Se-depleted HepG2 cells. The results show that the GPX enzyme activity essentially requires Se and that IL-6 further increases the Se-mediated GPX enzyme activity (Figure 14 A). In line with this, IL-6 slightly induced the GPX enzyme activity in Hep3B (Figure 14 B) and primary murine hepatocytes (Figure 14 C).

To study in more detail a potential GPX isozyme-specific regulation by IL-6, the protein expression levels of the GPX isozymes GPX1, GPX2 and GPX4 were examined via Western blot analyses (Figure 14 D). The expression of the GPX was induced by supplemental Se, while the addition of IL-6 regulated the GPX expression in an isozyme-specific manner. GPX2 protein expression was increased by IL-6, whereas GPX4 was slightly down-regulated, and GPX1 showed no response.

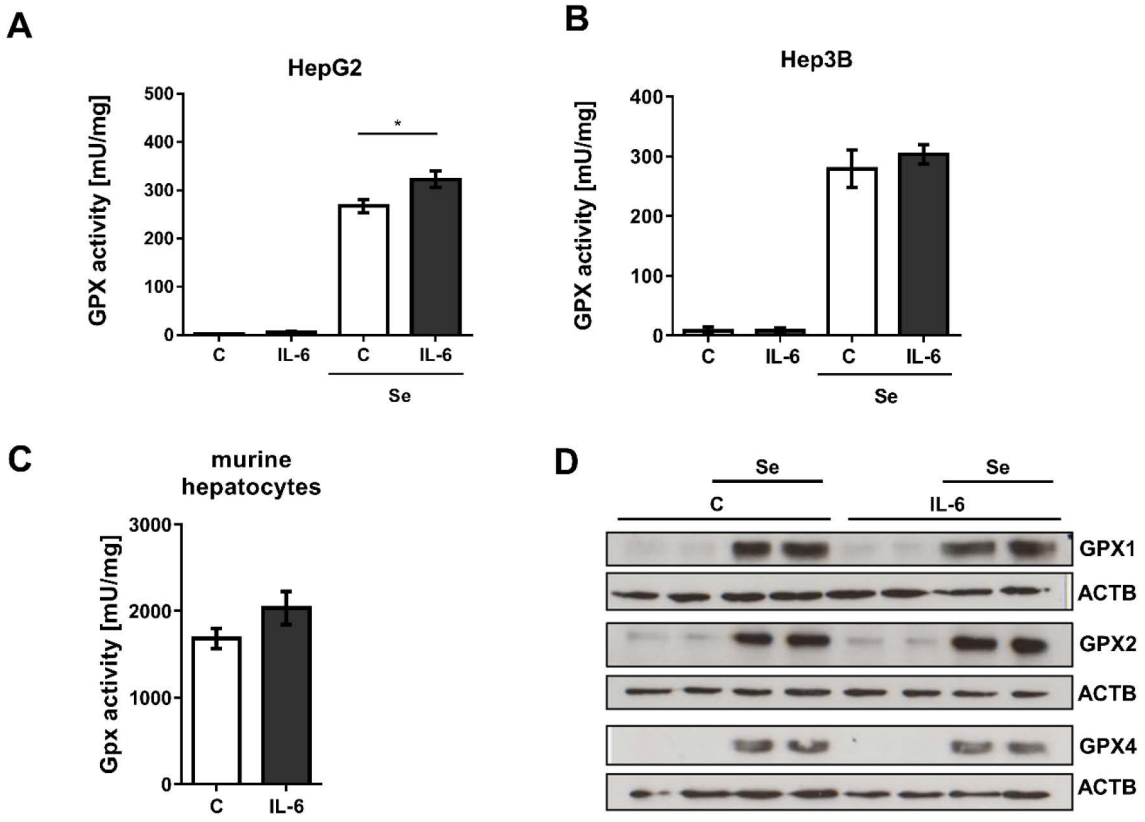


Figure 14: IL-6 regulates the GPX expression in hepatocytes.

(A) HepG2 cells were stimulated with IL-6 for 48 hours in the pre- or absence of Se and the total GPX enzyme activity was measured in cell lysates. A sufficient Se-supplementation in form of 100 nM sodium selenite is essential for the GPX enzyme activity. The GPX enzyme activity is also increased in response to IL-6 (n=5). An increase of GPX enzyme activity was also observed in (B) Hep3B cells (n=5) and (C) primary murine hepatocytes (n=3). (D) Western blot analysis of HepG2 cells showed that IL-6 induced the GPX2 expression, while GPX4 was slightly repressed and GPX1 was not affected (Mean ± SEM).

The transcript levels of *GPX1*, *GPX2* and *GPX4* were analysed and the expression levels were found to mirror that of Western blot analyses. These quantitative RT-PCR analyses revealed that the transcript level of *GPX1* was strongly induced by Se, whereas IL-6-mediated effects on the *GPX1* transcript level were negligible (Figure 15 A). In comparison, the *GPX2* transcript levels were significantly increased by IL-6 (~3-fold as compared to the control), while supplemental Se had no effect (Figure 15 B). In line with this, the transcript level of the *GPX4* remained unaffected by Se, while IL-6 reduced the *GPX4* transcript level by > 50% when compared to the untreated cells (Figure 15 C).

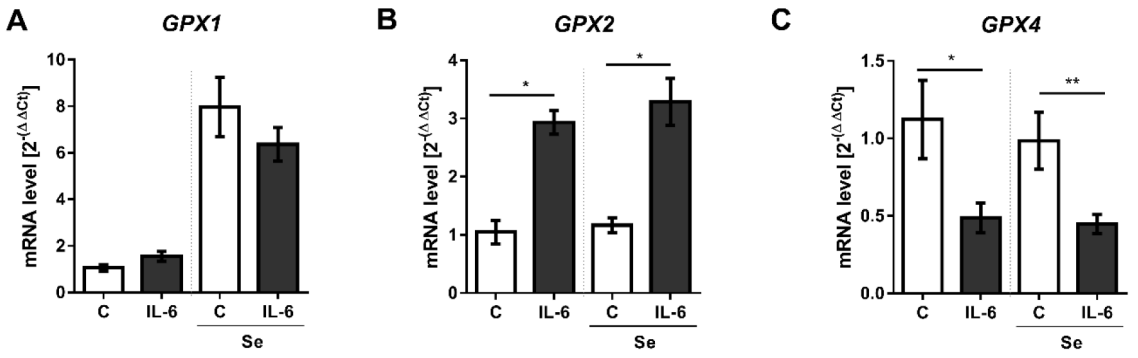


Figure 15: Isozyme-specific regulation of the GPX transcripts by IL-6.

Se-supplemented and Se-depleted HepG2 cells were stimulated for 24 hours with IL-6 and the transcript levels of *GPX1*, *GPX2* and *GPX4* were determined via quantitative RT-PCR. IL-6 regulated the GPX transcript levels in an isozyme-specific manner. (A) The *GPX1* transcript level was strongly induced by Se-supplementation, but an IL-6 mediated effect on the transcript level was negligible. (B) While the *GPX2* transcript level was significantly increased by IL-6, (C) the cytokine significantly reduced the *GPX4* transcript level by >50% compared to control. The transcript level of *GPX2* and *GPX4* remained unaffected by supplemental Se (Mean ± SEM, n=4-6).

To test whether IL-6 directly affects GPX expression via targeting their promoters, the regulation of IL-6 on the human-derived promoters of *GPX2* and *GPX4* was investigated. To investigate this, approximately 1000 bp of the promoter regions of *GPX2* and *GPX4* were cloned into FLuc-containing vectors and transiently transfected into HepG2 and Hep3B cells. The transfected HepG2 and Hep3B cells were stimulated with IL-6 for 24 hours and the promoter activity was analysed. (Figure 16). The data show that IL-6 significantly increased the *GPX2* promoter activity in both cell lines (Figure 16 A), whereas IL-6 strongly repressed the *GPX4* promoter activity (Figure 16 C).

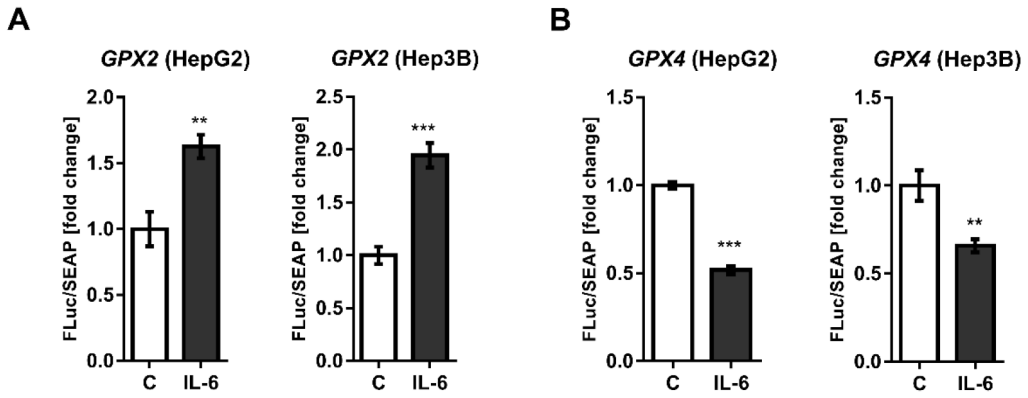


Figure 16: IL-6 regulates the GPX promoter activities in a gene-specific manner.

HepG2 and Hep3B cells were transiently transfected with approximately 1000 bp long promoter constructs derived from the human *GPX2* and *GPX4* genes. Cells were stimulated with IL-6 for 24 hours and changes in the promoter activity in response to IL-6 was analysed. (A) *GPX2* promoter activity was significantly induced by IL-6 in both cell lines. (B) IL-6 markedly repressed *GPX4* promoter in HepG2 and Hep3B cells (Mean ± SEM, n=4-6).

TXNRD is a second family of selenoproteins that are involved in antioxidative defence and catalyse the reduction of the antioxidant thioredoxin. The effects of IL-6 on the TXNRD expression was investigated in parallel to GPX. However, in these analyses no consistent evidence for TXNRD regulation by IL-6 was found (data not shown).

Selenoproteins are also involved in the thyroid hormone metabolism in the form of DIO, which catalyse the inactivation and activation of the thyroid hormones (TH) by deiodination. The thyroid mainly secretes the inactive TH T4 that becomes activated to active T3 by DIO1 primarily expressed in liver. Non-thyroidal illness syndrome becomes apparent in seriously ill patients and is characterized by low fT3, and if persistent low fT4 serum level. Lipopolysaccharide (LPS)-induced NTIS serves as a model for septic shock. This model has shown that NTIS impairs the DIO1 expression in liver and kidney [Castro, et al., 2013]. As LPS-induces the biosynthesis of IL-6 [Beurel and Jope, 2009], it was a clear question as to whether IL-6 has a negative impact on DIO1 expression in hepatocytes.

To investigate DIO1 expression in response to IL-6, HepG2 cells were treated with IL-6 and the regulation of DIO1 was quantified by: enzyme activity assay, Western blot analysis, quantitative RT-PCR and *DIO1* reporter gene assay (Figure 17).

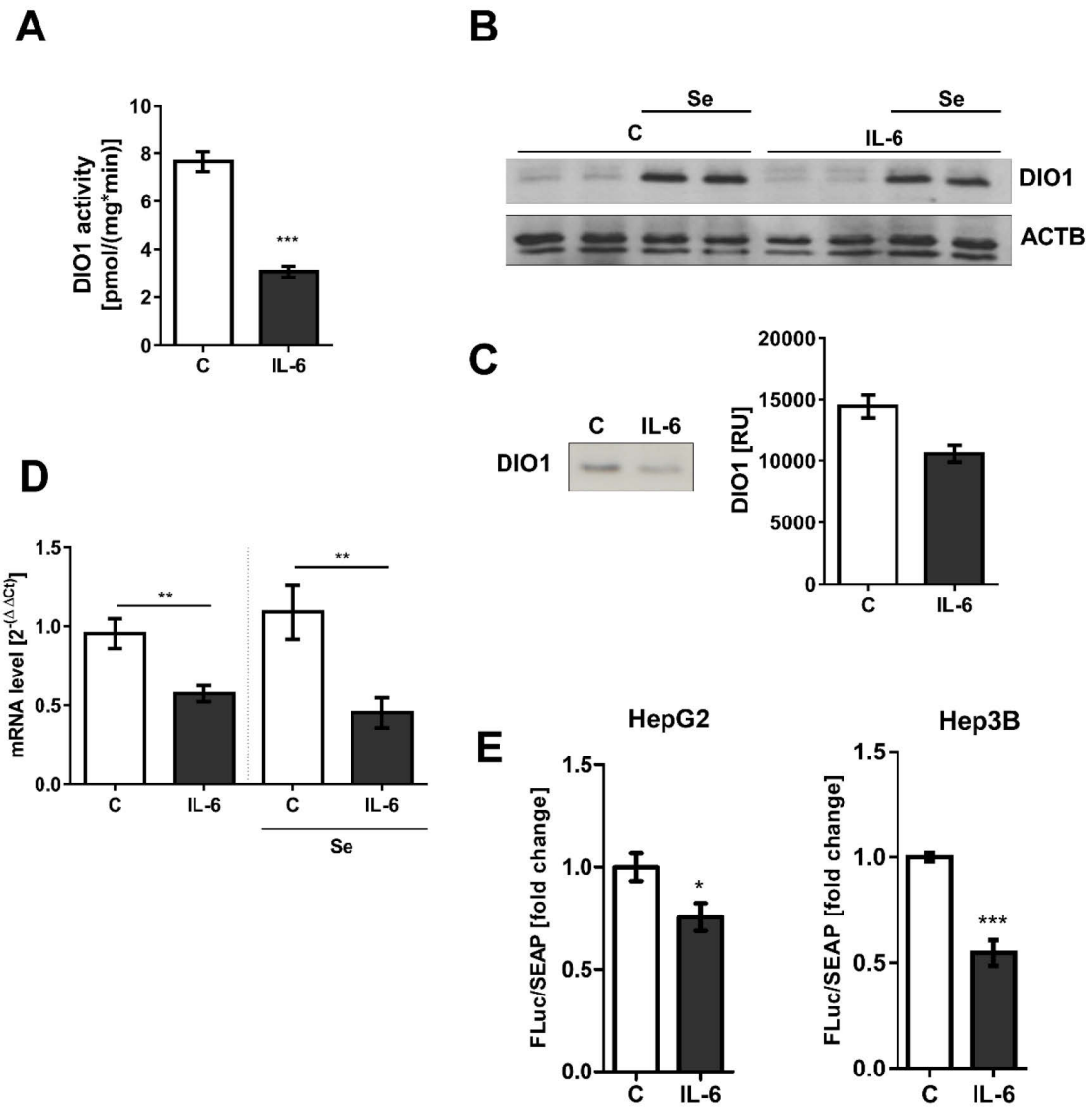


Figure 17: IL-6 down-regulates DIO1 expression.

Se-supplemented and Se-depleted HepG2 cells were treated with IL-6 for 24 and 48 hours, respectively. (A) The DIO1 enzyme activity was strongly diminished by IL-6 by more than 50% in comparison to control (n=6). In line with these findings, a reduction of DIO1 expression was observed in (B) Western blot analysis, and (C) quantitative Western blot evaluation using ImageJ (n=4). In parallel, a significant decreased *DIO1* transcript level was detected (D), regardless of a Se-supplementation (n=6). (E) The *DIO1* promoter activity was significantly reduced in response to IL-6 in in HepG2 and Hep3B cells (n=6) (Mean ± SEM).

DIO1 enzyme activity was strongly reduced by IL-6 by more than 50% when compared to the control (Figure 17 A). A clear reduction of DIO1 expression was also observed at protein level (Figure 17 B+C), and transcript level (Figure 17 D). Furthermore, the *DIO1* promoter activity was greatly reduced by HepG2 and Hep3B cells. Taken together these results indicate IL-6-mediated down-regulation of DIO1 occurs directly via the *DIO1* promoter (Figure 17 E).

3.2. Synergistic effects of pro-inflammatory cytokines on selenoprotein expression

The acute phase response (APR) is highly orchestrated by the immune system to fight infection, inflammation or tissue injury. APR involves the elevated expression of various pro-inflammatory cytokines including IL-6, interleukin-1β (IL-1β) and tumour necrosis factor α (TNFα). These cytokines act as mediators of the APR and promote the secretion of acute phase proteins (e.g. CRP or CP) primarily by the liver [Moshage, 1997]. Both IL-1β and TNFα lead to a strong production of IL-6 from a variety of cell types, e.g. endothelial cells. As it is known that IL-6 is able to mediate the regulation of selenoprotein expression, a possible synergistic effect of IL-6 with IL-1β and TNFα on selenoprotein expression and activity was investigated.

To this end, HepG2 cells were treated for 48 hours with IL-6 (100 ng/mL), IL-1 β (25 ng/mL) and TNFα (50 ng/mL) individually or in combination. The investigation of selenoprotein expression was carried out using Western blot analyses and immunoassay of SEPP as similarly described in section 3.1. The isolated application of IL-1β and TNFα slightly induced the SEPP protein expression, while a single or combined stimulation with IL-6 strongly reduced the SEPP expression (Figure 18 A). Notably, stimulation with IL-6 in combination with either IL-1β, TNFα or both similarly suppressed the SEPP expression as IL-6 alone. In Hep3B cells, the SEPP expression was slightly reduced by IL-6, TNFα or in combination of both. In comparison, no effect on the SEPP expression was observed for cells treated with IL-1β alone (Figure 18 B).

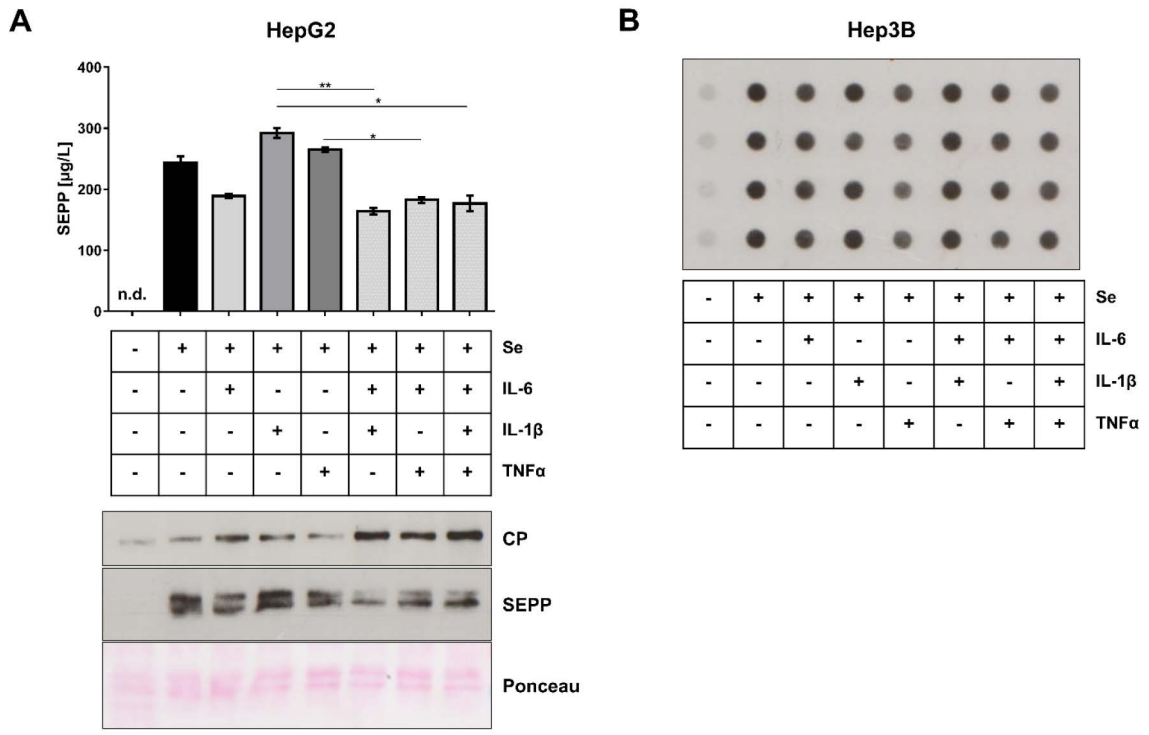


Figure 18: Synergistic effects of IL-6, IL-1β and TNFα on SEPP expression.

HepG2 and Hep3B cells were stimulated for 48 hours with IL-6, IL-1β and TNFα alone or in combination. The SEPP expression was determined from cell culture medium using Western blot analyses and SEPP-ELISA. (A) In HepG2 cells, isolated application of IL-1β and TNFα induced the SEPP expression, while a single or combined stimulation with IL-6 strongly reduced the SEPP expression. (B) In Hep3B cells, SEPP expression was slightly reduced by IL-6, TNFα alone or in combination. No reduction in SEPP expression was observed for IL-1β (Mean ±SEM, n=4).

The following investigations examined the synergistic effects of IL-6, IL-1β and TNFα on the absolute GPX enzyme activity. Se-supplemented HepG2, Hep3B and primary murine hepatocyte cells were stimulated with IL-6, IL-1β and TNFα for 48 hours. The results show that the GPX enzyme activity in HepG2 cells was enhanced by IL-6, IL-1β and TNFα in comparison to Se-control (Figure 19 A). A much stronger induction of the GPX enzyme activity was observed with a combined stimulation with IL-6, IL-1β and TNFα. Subsequent Western blot analysis of GPX4 showed a slight reduction when treated with IL-1β alone or in combination with IL-6 and/or TNFα as compared to Se-control. However, the absolute GPX enzyme activity was markedly increased, despite a down-regulation of different GPX isozymes mediated by individual cytokines as described in section 3.1.2. In line with these findings, a combined stimulation with IL-6, IL-1β and TNFα significantly induced the GPX enzyme activity in Hep3B cells (Figure 19 B). In primary murine hepatocytes, the combination of IL-6, IL-1β and TNFα had no significant effect (Figure 19 C).

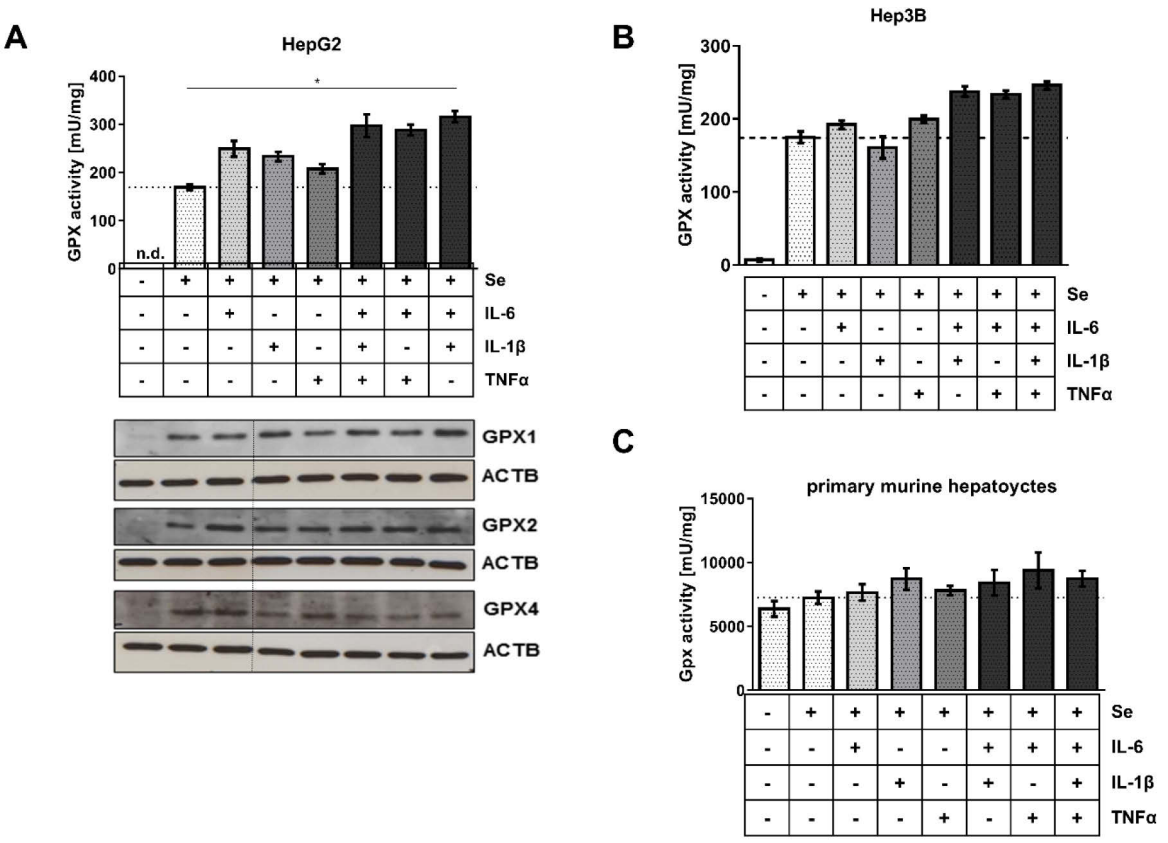


Figure 19: Synergistic effects of IL-6, IL-1β and TNFα on GPX expression.

HepG2, Hep3B and primary murine hepatocyte cells were treated alone or in combination with IL-6, IL-1β and TNFα for 48 hours. (A) In HepG2 cells, GPX enzyme activity was induced by IL-6, IL-1β and TNFα when compared to the Se-control. A combined stimulation with these cytokines resulted in a stronger induction in GPX enzyme activity as compared to a single application. Western blot analysis of GPX4 showed a slight reduction when treated with IL-1β or in combination with IL-6 and TNFα when compared to Se-control. (B) A single application of IL-6, IL-1β and TNFα did not affect GPX enzyme activity in Hep3B cells, whereas cytokine combination induced the activity significantly. (C) In primary murine hepatocytes, single of combined application of IL-6, IL-1β and TNFα had no significant effect on Gpx enzyme activity (Mean ± SEM, n=3-4).

It has been previously reported that DIO1 is down-regulated by different pro-inflammatory cytokines [Jakobs, et al., 2002; Xu, et al., 2014]. However, combined effects of IL-6, IL-1β and TNFα have not yet been studied. To investigate this relationship, we tested whether these cytokines have a synergistic effect on the expression of DIO1. Furthermore, the extent to which combination of different pro-inflammatory cytokines might promote an IL-6 mediated down-regulation of DIO1 was investigated (Figure 20). Se-supplemented HepG2 cells were stimulated for 48 hours with IL-6, IL-1β and TNFα and DIO1 enzyme activity assay and Western blot analysis was used to determine the impact on DIO1 expression. While TNFα did not affect the DIO1 expression, the cytokines IL-6 and IL-1β strongly repressed the DIO1 protein level and accordingly the DIO1 enzyme activity. More interestingly, a combined

stimulation with IL-6, IL-1β and TNFα repressed DIO1 protein and enzyme activity in an additive-like manner by more than 50% when compared to the Se-control.

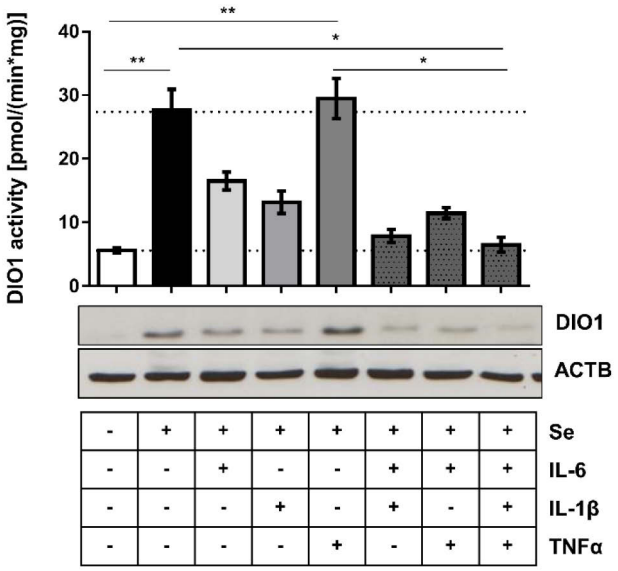


Figure 20: IL-6, IL-1β and TNFα repress DIO1 expression in an additive-like manner.

HepG2 cells were treated alone or in combination with IL-6, IL-1β and TNFα, and DIO1 enzyme activity and protein expression were analysed. Compared to the Se-control, TNFα did not affect the DIO1 expression. IL-6 and IL-1β however, strongly repressed the DIO1 protein level and enzyme activity. Combining IL-6, IL-1β and TNFα repressed the DIO1 protein expression and enzyme activity in an additive-like manner (Mean ± SEM, n=4).

3.3. Regulation of selenoprotein expression by AG

3.3.1. AG-induced UGA codon readthrough in selenoprotein translation

AG negatively interfere with the mRNA proofreading process leading to misinsertion of amino acids, increasing error rates in protein synthesis and possible production of non-functional proteins. Importantly for selenoproteins, AG affect the UGA codon mandatory for incorporation of Sec-residues into selenoproteins. In addition to the UGA codon, each selenoprotein gene contains a unique SECIS-element in the 3' UTR of the mRNA that is required for a successful incorporation of Sec-residues. Therefore, it was hypothesised that the AG-mediated misinterpretation of UGA codon and the Sec-incorporation machinery directly interfere with each other. To study the nature of this interference, Se-dependent reporter constructs were developed. These reporter constructs encode for a fusion protein of FLuc and RLuc, separated by a UGA codon allowing studies on: a) Sec-insertion b) UGA misinterpretation or c) early termination. These constructs were then stably transfected into the artificial cell model HEK293 and a constant expression of all reporter cassettes was verified.

From the known set of selenoprotein genes, different SECIS-elements were selected including one being strictly Se-responsive (*GPX1*), another being crucial and favourably Se-supplied (*GPX4*), or the one containing two separate SECIS-elements (*SEPP*). A SECIS-free reporter and 100% readthrough reporter were used as control. The SECIS-free reporter contains an UGA codon but no SECIS-element. The 100% readthrough reporter contains no stop codon guaranteeing an unaffected readthrough. The functionality of these reporter constructs was tested as response to increasing concentrations of Se (Figure 21).

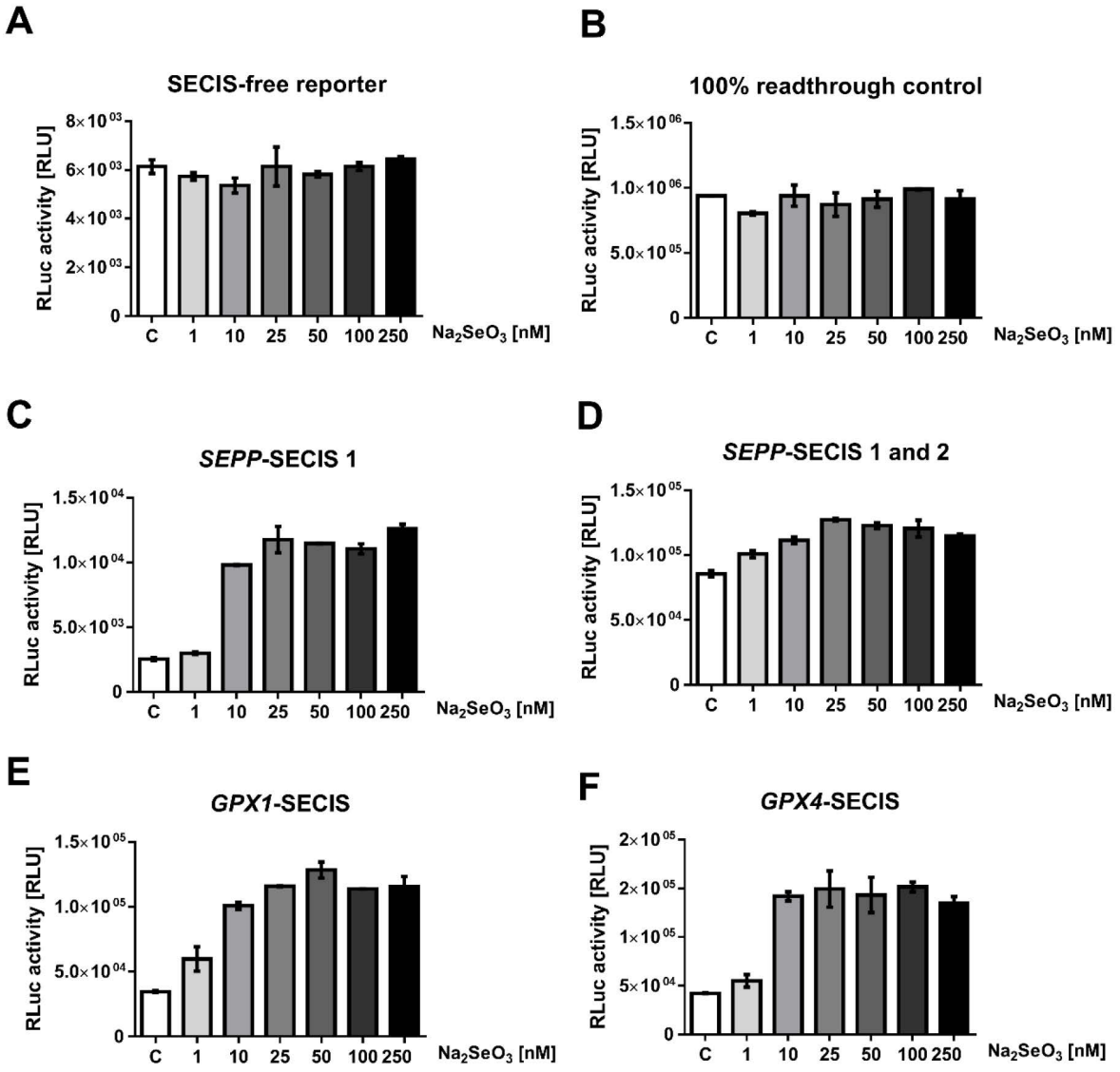


Figure 21: UGA readthrough efficiency is SECIS-element dependent.

The Se-response of Se-dependent reporter constructs was tested in a dose-dependent manner (0 to 250 nM Na_2SeO_3). No Se-dependent reporter activity was observed for the (A) SECIS-free reporter, and (B) the 100% readthrough control. In contrast, a variable Se-dependent increase was detected for all SECIS-elements tested; some marginal Se-dependent increase of (D) *SEPP*-SECIS1&2 and (E) *GPX1*-SECIS, and a strong increase of (E) *SEPP*-SECIS1 and (D) *GPX4*-SECIS (Mean ± SEM, n = 2–3).

Both control reporters, the SECIS-free and 100% readthrough reporter, remained unaffected to increasing Se-concentrations (0-200 nM Na_2SeO_3), however basal activity intensely varied between the two reporters (Figure 21 A+B). The Se-dependent reporters responded to Se in a dose-dependent manner, although the extent of response varied between reporters (Figure 21 C-G). In detail, the reporter containing the first SECIS-element of *SEPP* (*SEPP*-SECIS1) showed a strong response to Se, reaching a maximal response to supplemental Se already at

10 nM sodium selenite. In comparison, a combination of both *SEPP*-SECIS-elements (*SEPP*-SECIS1&2) resulted in an only marginal sensitivity to increasing Se-concentrations. However, the overall translation level of *SEPP*-SECIS1&2 was much higher as compared to *SEPP*-SECIS1 alone (Figure 21 C, D). The Se-dependent reporter derived from the Se-sensitive *GPX1* gene responded more gradual in a concentration range from 0 to 50 nM sodium selenite (Figure 21 E). As opposed to that, the *GPX4*-SECIS construct derived from the essential selenoprotein *GPX4* gene exhibited a similar rapid and steep response as compared to the *SEPP*-SECIS1 reporter (Figure 21 F).

In order to identify AG impairing the Se-mediated UGA readthrough efficiency in selenoprotein translation, a set of different AG was tested for their UGA readthrough efficiency in SECIS-free reporters. To this end, stably transfected HEK293 cells were stimulated with each of two concentrations of different AG, and Se for 48 hours (Figure 22).

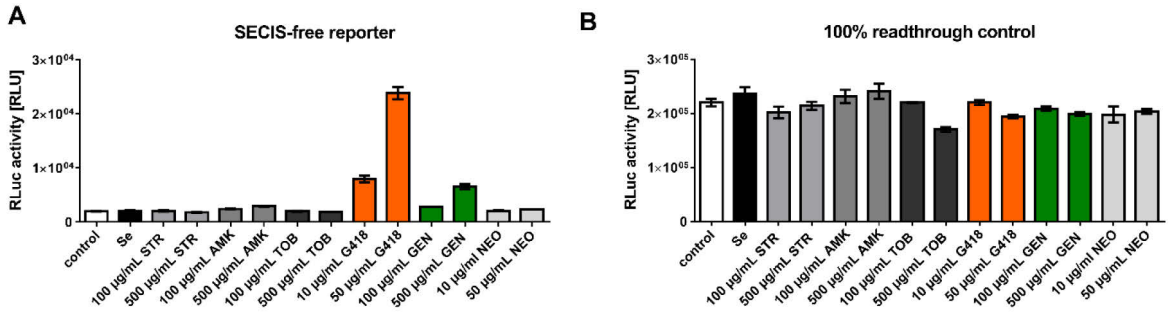


Figure 22: AG potentially impairing UGA readthrough efficiency.

Stably transfected HEK293 cells were tested for their effects on UGA readthrough efficiency in SECIS-free and 100% readthrough control reporters. (A) The SECIS-free reporter (containing a UGA codon) showed a strong dose-dependent response to G418, and to a lesser extent to gentamicin. (B) The 100% readthrough control reporter exhibited no effect during AG treatment. (Abbreviation: C= control, STR =streptomycin, AMK = amikacin, TOB = tobramycin, G418 = geneticin, GEN = gentamicin, NEO = neomycin (Mean ± SEM, n=3).

While most AG, i.e., streptomycin, amikacin, tobramycin or neomycin did not affect UGA readthrough in the SECIS-free reporter, the AG geneticin (G418) strongly induced the UGA readthrough in a concentration-dependent manner (Figure 22 A). In addition, the AG gentamicin, often used in clinical antibiotic therapy, showed a slight dose-dependent induction in the UGA readthrough assay. In comparison, the 100% readthrough control vector showed no response to AG (Figure 22 B). Following these results, further experiments focused on the effects of the AG G418 and gentamicin on the UGA readthrough efficiency in Se-dependent reporter constructs.

The previously described, stably transfected HEK293 cells were stimulated with two different concentrations of G418 (10 and 50 µg/mL) or gentamicin (100 and 500 µg/mL), and the effects were compared to the Se-control (100 nM Na_2SeO_3). Cell viability test using the MTT-test did not show any signs of cell toxicity in the applied AG concentrations.

While the 100% readthrough control reporter was not affected by any of the two AG concentrations, the negative reporter was strongly induced by G418 and to a lesser extent by gentamicin, in a dose-dependent manner. Both control reporters remained unaffected to supplemental Se (Figure 23 A+B). In comparison to the four Se-dependent reporter constructs analysed above, some fundamental differences in the response to G418 and gentamicin were observed. The *SEPP*-based SECIS-element constructs (*SEPP*-SECIS1 and *SEPP*-SECIS1&2) markedly differed in their response to AG. While the *SEPP*-SECIS1 reporter strongly responded to Se and G418 (Figure 23 C), the *SEPP*-SECIS1&2-element remained relatively unaffected (Figure 23 D). Both *SEPP*-SECIS-element constructs responded to AG in a similar manner as to supplemental Se (Figure 23 C, D). In comparison, the *GPX1*-SECIS-element showed similarly high responses to 100 nM sodium selenite and to 10 or 50 µg/mL G418 (Figure 23 E). The *GPX4*-SECIS reporter displayed a high sensitivity to the AG-mediated UGA readthrough (Figure 23 F). The response of the *GPX4*-SECIS-element to 50 µg/mL G418 was even higher than in response to Se-supplementation. The application of gentamicin did not affect any of the Se-dependent reporter constructs.

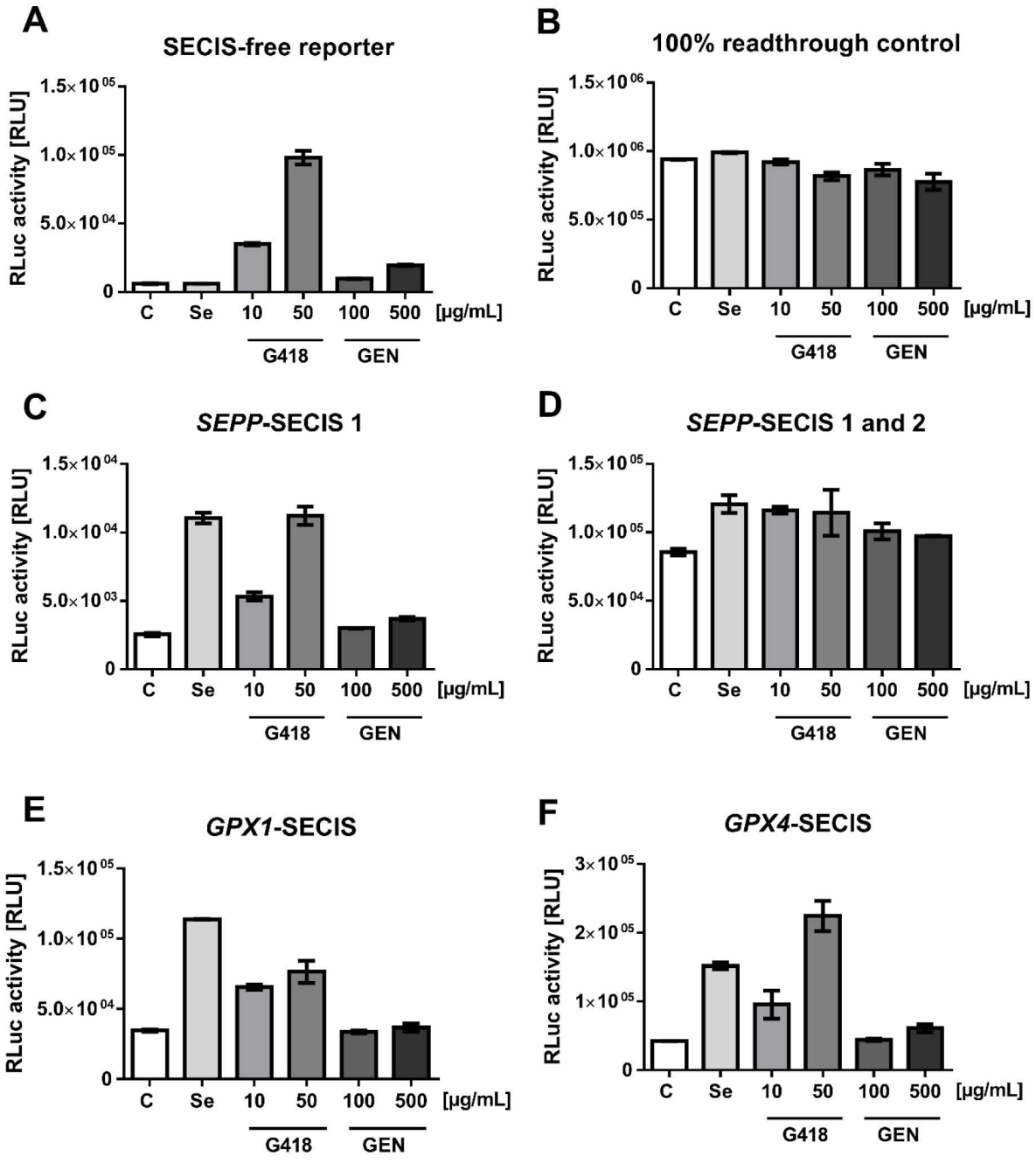


Figure 23: AG-induced UGA readthrough in Se-dependent reporters.

Se-dependent and control reporters were tested with respect to AG-dependent UGA readthrough. (A) While the UGA readthrough of the AG-sensitive SECIS-free reporter was strongly induced in a dose-dependent manner, (B) no AG-driven UGA readthrough was found for the 100% readthrough reporter. A strong G418- and lower gentamicin response was also observed for (C) *SEPP*-SECIS1, (E) *GPX1*-SECIS and (F) *GPX4*-SECIS reporter construct. The response of (D) *SEPP*-SECIS1&2 reporter construct to G418 and gentamicin was relatively low (Mean ± SEM, n = 2–3).

To further investigate whether Se might modulate the AG-induced UGA readthrough efficiency, stably transfected HEK293 cells were stimulated with G418 alone, or in combination with supplemental Se. The combined effect of Se and G418 was analysed as fold change calculated to the stimulation controls (Figure 24). While a modulatory effect of supplemental Se on the UGA readthrough efficiency was observed for the G418-treated SECIS-free control reporter (Figure 24 A), a co-stimulation with Se markedly reduced the G418-driven UGA readthrough of all Se-dependent SECIS reporters (Figure 24 B-D). The G418-induced UGA readthrough of the Se-sensitive *GPX1*-SECIS reporter declined from 1.6-fold to 0.8-fold under Se-supplementation (Figure 24 B).

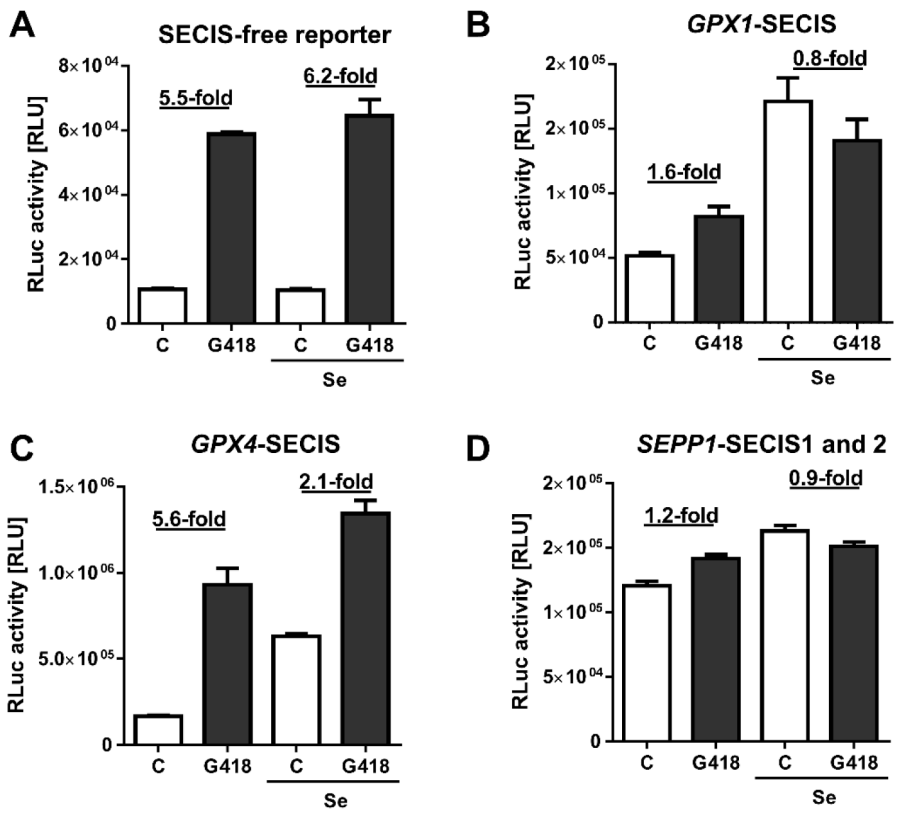


Figure 24: Synergistic effects of G418 and Se on Se-dependent reporters.

To investigate whether supplemental Se alters G418-driven UGA readthrough of the SECIS-element reporters, a co-stimulation experiment with Se and G418 was performed. (A) No Se-mediated modulatory effect on UGA readthrough was observed for the SECIS-free control reporter. By contrast, a SECIS-element dependent decline in G418-induced UGA readthrough upon Se-supplementation was detected for (B) *GPX1*-SECIS, (C) *GPX4*-SECIS and to a lesser extent for (D) *SEPP*-SECIS1&2 (Mean ± SEM; n = 2-3).

To concur with the previous findings, the application of Se in combination with G418 reduced the G418-induced UGA readthrough of the *GPX4*-SECIS reporter (Figure 24 C; 5.6-fold to 2.1-fold) and to a lower extent of the less Se-sensitive *SEPP*-SECIS1&2 reporter (Figure 24 D; 1.2-fold to 0.9-fold). Overall, the expression levels of all SECIS-element reporters were much higher in the presence of Se. These findings clearly suggest that the nature of the SECIS-element defines whether and to what extent supplemental Se modulates the G418-mediated UGA readthrough.

3.3.2. AG-induced modulation of hepatic selenoprotein expression

The findings on the AG-induced UGA readthrough (described in section 3.3.1) indicate that the overall effect of G418 and gentamicin on selenoprotein translation relies on the interplay of various factors including the type of AG, the AG-concentration, the SECIS-element and the Se-availability [Martitz, et al., 2016]. These results lead to the question what impact can AG have on the expression of selenoproteins and the enzymatic activity of selenoenzymes?

AG-driven effects on SEPP expression

The Se-transporter SEPP contains two different SECIS-elements and ten UGA codons. This high number of UGA codons my lead to SEPP being highly sensitive to AG-induced UGA-readthrough. As SEPP concentration declines during inflammatory diseases and negatively correlates with the outcome of severe sepsis, the additional application of AG may result in the production of Se-deficient or even Se-free SEPP worsening the Se-deficiency of peripheral SEPP target organs. In order to investigate AG effects on SEPP expression, human and murine hepatoma cells lines were stimulated with several concentrations of G418 and gentamicin for 48 hours. Western blot analysis of Se-depleted HepG2 cells showed that the intra- and extracellular SEPP expression was strongly induced by G418 in a concentration-dependent manner (Figure 25 A), and to lesser extent by gentamicin (Figure 25 B). Compared to the strong inductive effects of G418, SEPP expression was only moderately promoted by gentamicin. For this reason, the effect of higher gentamicin concentrations was tested (Figure 25 B). Higher gentamicin concentrations induced the SEPP expression in a concentration-dependent manner; however, the increases were lower than in comparison to supplemental Se. In line with this, G418 strongly induced the SEPP expression in Hep3B (Figure 25 C) and the murine Hepa1-6 cells (Figure 25 D).

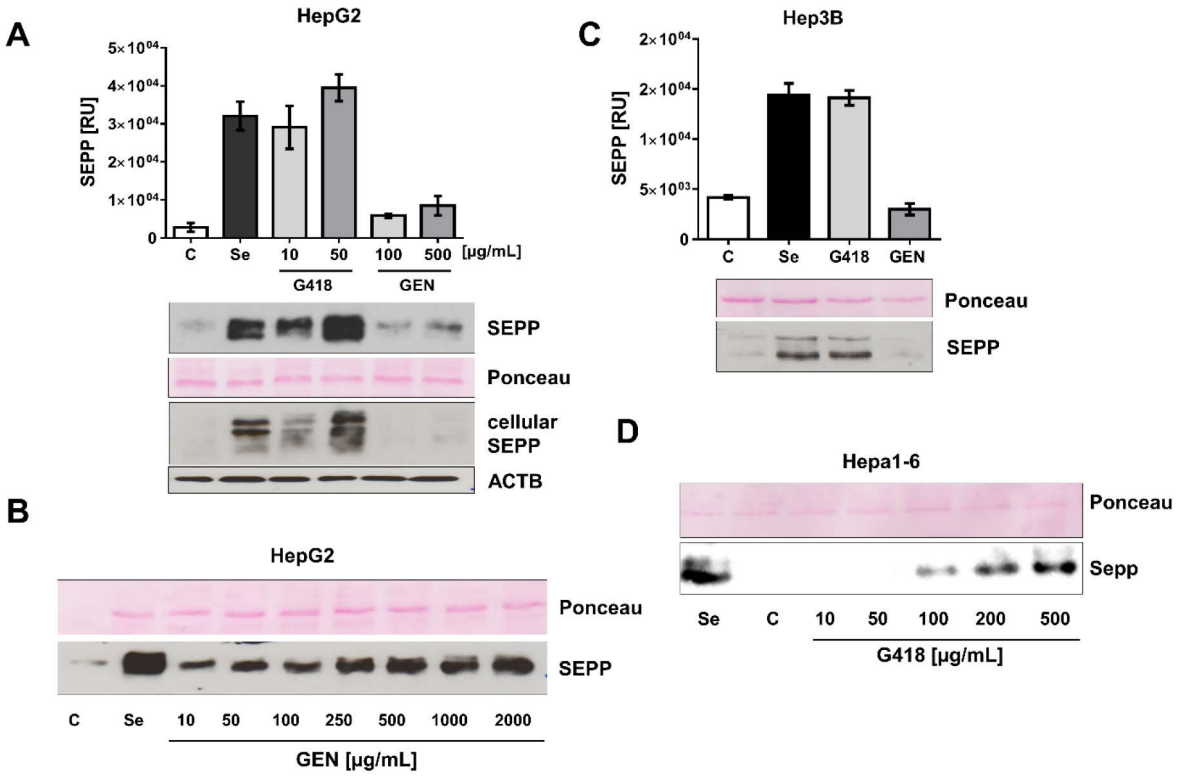


Figure 25: AG increase SEPP expression in hepatoma cell lines.

Human and murine hepatoma cell lines were stimulated with G418, gentamicin or Se. The expression of SEPP was analysed via Western blot analysis and densitometrically analysed using the software ImageJ. (A) In HepG2 cells, G418 significantly increased the SEPP expression in a dose-dependent manner. Gentamicin increased the SEPP expression, but to a lesser extent as compared to G418. (B) Higher concentrations of gentamicin strongly induced SEPP expression in a dose dependent manner, but remained lower in comparison to cells supplemented with Se. (C) In Hep3B cells, G418 but not gentamicin intensely promoted the SEPP expression equally to Se. (D) G418 also induced the Sepp expression in murine Hepa1-6 cells in a dose-dependent manner (Mean ± SEM, n=3).

However, much higher concentrations of G418 were required to induce Sepp expression in murine Hepa1-6 cells as compared to the human HepG2 and Hep3B cell lines.

In order to verify these findings, a quantitative analysis of SEPP was performed. G418 strongly increased the SEPP concentration in a dose-dependent manner and independent of supplemental Se (Figure 26 A).

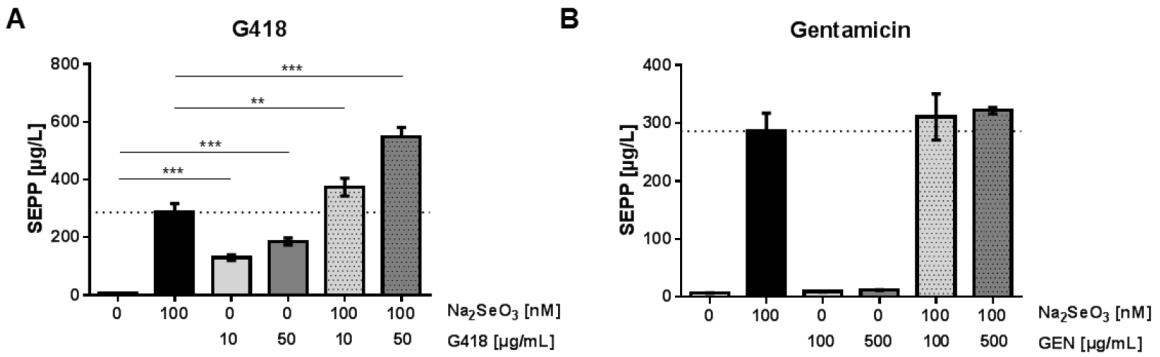


Figure 26: Synergistic effects of G418, gentamicin and Se on SEPP concentration.

HepG2 cells were treated for 48 hours with G418 or gentamicin, and Se. Quantitative analysis of SEPP concentration was investigated using SEPP-ELISA. (A) G418 strongly increased the SEPP level in a dose-dependent manner, independent of supplemental Se. Co-stimulation with sodium selenite indicated a synergistic effect in the SEPP expression. (B) Gentamicin did not promote the expression of SEPP (Mean ± SEM, n=6).

However, the combined stimulation with Se increased the SEPP concentration to a greater extent when compared to G418 or Se alone. This outcome implies a synergistic effect of Se and G418 on SEPP biosynthesis. In comparison, gentamicin had no effect on the SEPP concentration (Figure 26 B).

As AG interfere with the small ribosomal subunit required for transcription, the question arose whether AG may directly affect the gene expression of selenoproteins. To answer this point, HepG2 cells were stimulated with two concentrations of G418 (10 and 50 µg/mL), gentamicin (100 and 500 µg/mL) and Se. The regulation of gene expression was then analysed by quantitative RT-PCR (Figure 27). The DNA damage-inducible transcript 3 (*DDIT3)* has recently been identified as an AG-sensitive transcript and was used as positive AG-control in all experiments [Tao and Segil, 2015]. DDIT3 induces i.a. cell cycle arrest and apoptosis in response to ER stress. *DDIT3* showed a dose-dependent induction in transcript level for G418, and minor for gentamicin (Figure 27 A). An application of 50 µg/mL G418 resulted in an extraordinarily strong induction of *DDIT3* (12-fold as compared to negative control). It is interesting to note that G418 and Se increased the *SEPP* transcript level in a similar manner (G418; 4.4-fold above control versus Se; 4.1-fold above control), while a gentamicin-mediated effects were negligible (Figure 27 B). Collectively, these findings show that AG affect the *SEPP* transcript level and SEPP protein level.

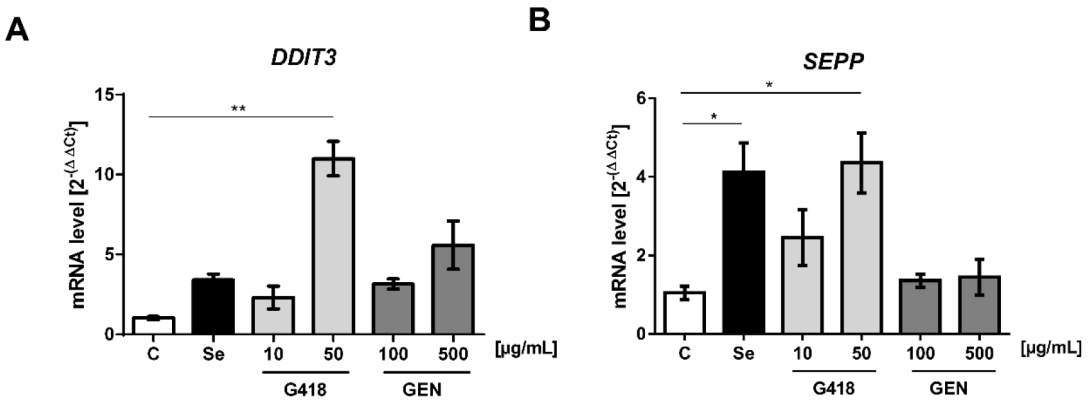


Figure 27: G418 and gentamicin affect the *SEPP* transcript level.

HepG2 cells were stimulated with 10 or 50 µg/mL G418, 100 or 500 µg/mL gentamicin, and Se for 24 hours. The SEPP transcript level was analysed using quantitative RT-PCR. (A) The DNA damage-inducible transcript 3 (*DDIT3*) used as positive control for AG stimulation, showed a dose-dependent induction in transcript level for both, G418 and gentamicin. 50 µg/mL G418 led to an extraordinarily strong induction of the *DDIT3* transcript level (12-fold compared to negative control). (B) G418 and Se strongly increased the *SEPP* transcript level in a comparable manner; while gentamicin had no significant effect (Mean ± SEM, n=4).

In a next step, we wanted to test whether the AG-induced misinterpretation of UGA codon leads to the insertion of alternative amino acids resulting in the biosynthesis of Se-poor SEPP isoforms. For this reason, conditioned media of Se- or AG-stimulated HepG2 cells were collected and SEPP was immobilised on a size-defined nitrocellulose membrane using Dot blot technique and the SEPP bound Se was quantified as described in section 2.17 (Figure 28). In line with the previous results, Se (100 nM Na_2SeO_3) or G418 (100 µg/mL) induced SEPP biosynthesis in a similar manner (Figure 28 A). The combined application of G418 with increasing Se-concentrations led to a significant and additive rise in total SEPP-concentration in comparison to a single application of G418 or Se.

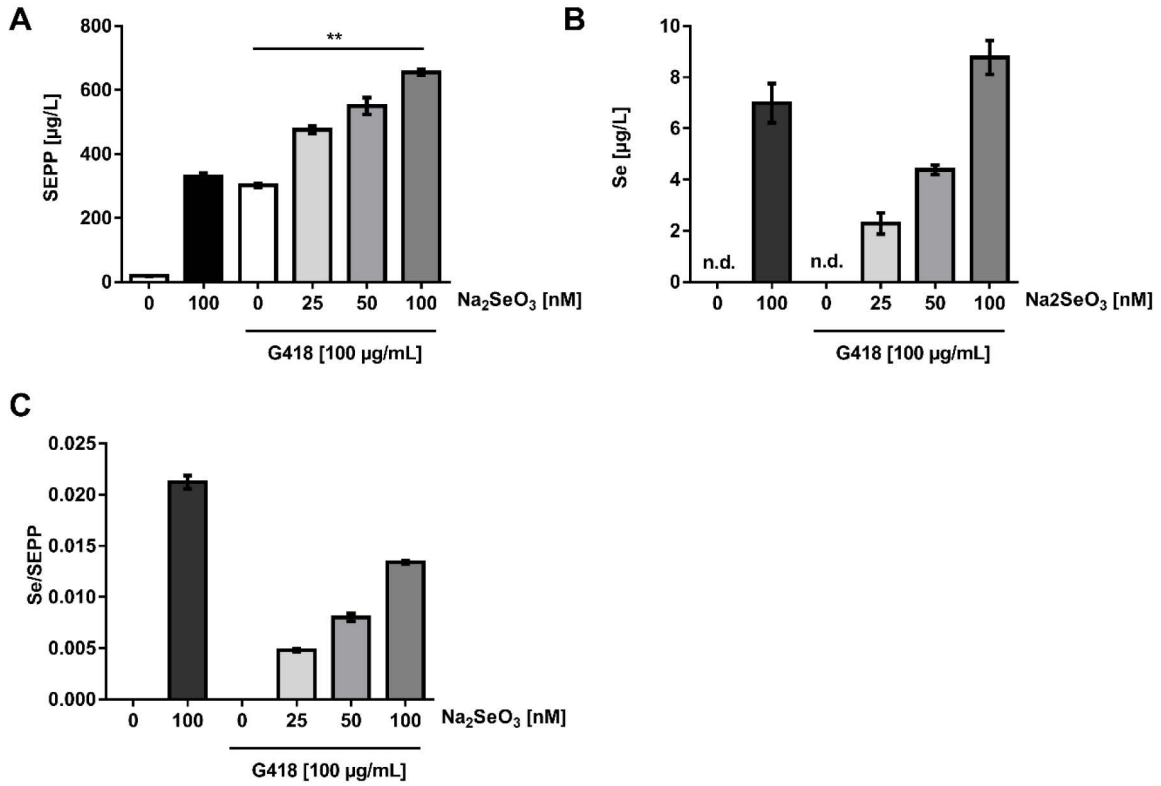


Figure 28: G418 promotes the biosynthesis of Se-deficient SEPP.

HepG2 cells were treated with G418, Se (100 nM sodium selenite) or G418 co-supplemented with 25, 50 or 100 nM sodium selenite. (A) SEPP concentrations indicate that G418 and Se individually induced increases of SEPP biosynthesis to a similar extent, whereas G418 co-supplemented with Se resulted in a significant and additive increase in SEPP expression. (B) No Se was detected in the immobilised proteins of cells left untreated or supplemented with G418. Co-simulation of G418 with increasing concentrations of Se led to an increase of Se-content of the immobilised proteins in a dose-dependent manner and even higher than Se- or G418-supplementation alone. (C) The molar ratio of Se per SEPP showed that G418-induced SEPP production contains no Se, but that supplemental Se was able to overcome this negative effect (Mean ± SEM, n=3).

The analysis of Se-content indicated that the immobilised proteins isolated from untreated or G418-treated HepG2 cells had no detectable Se (Figure 28 B). A co-supplementation with increasing Se-concentrations enlarged the Se-concentration in the immobilised proteins, irrespective of G418 presence. Finally, the molar ratio of Se per SEPP (Se/SEPP) demonstrated that SEPP synthesised by G418-treated cells was largely devoid of any Se. It is interesting to note that the G418-induced lack of Se-incorporation into SEPP was overcome by supplementing with Se. This results indicate that AG-induced UGA misinterpretation and failure of Sec-insertion can be prevented through Se-supplementation (Figure 28 C).

AG-mediated effects on hepatic GPX and DIO1

The previous investigations on AG-induced UGA codon readthrough of the selenoproteins SEPP, GPX1 and GPX4 have illustrated a SECIS-element-specific response to AG and Se. While the SECIS-element that originated from the Se-sensitive *GPX1* gene appeared to be highly AG-sensitive, the *GPX4*-derived SECIS-element was less dynamic. With this disparity in SECIS-response, we hypothesised that the AG-induced UGA readthrough also affects the expression and enzymatic activity of these selenoproteins in a similar isozyme-specific manner.

In order to prove this hypothesis, Se-supplemented and Se-depleted hepatocytes were stimulated with G418 (10 µg/mL or 50 µg/mL) and gentamicin (100 µg/mL or 500 µg/mL) for 48 hours and the changes in GPX expression levels were analysed. In line with earlier findings, G418, but not gentamicin induced the protein expression of GPX1, GPX2 and GPX4 (Figure 29 A). The G418-induced increase of GPX1 and GPX4 protein expression appeared to be more prominent in comparison to the GPX2 isozyme. However, G418 was unable to increase GPX expression to a similar extent to that of supplemental Se. Although in the Se-depleted HepG2 cells G418 slightly promoted the biosynthesis of GPX, GPX enzyme activity was not detectable (Figure 29 B). Similar findings were made in Se-depleted cells stimulated with gentamicin. However, when the HepG2 cells were stimulated with G418 or gentamicin in combination with Se, the GPX enzyme activity exhibited a similar level as Se-supplemented cells alone.

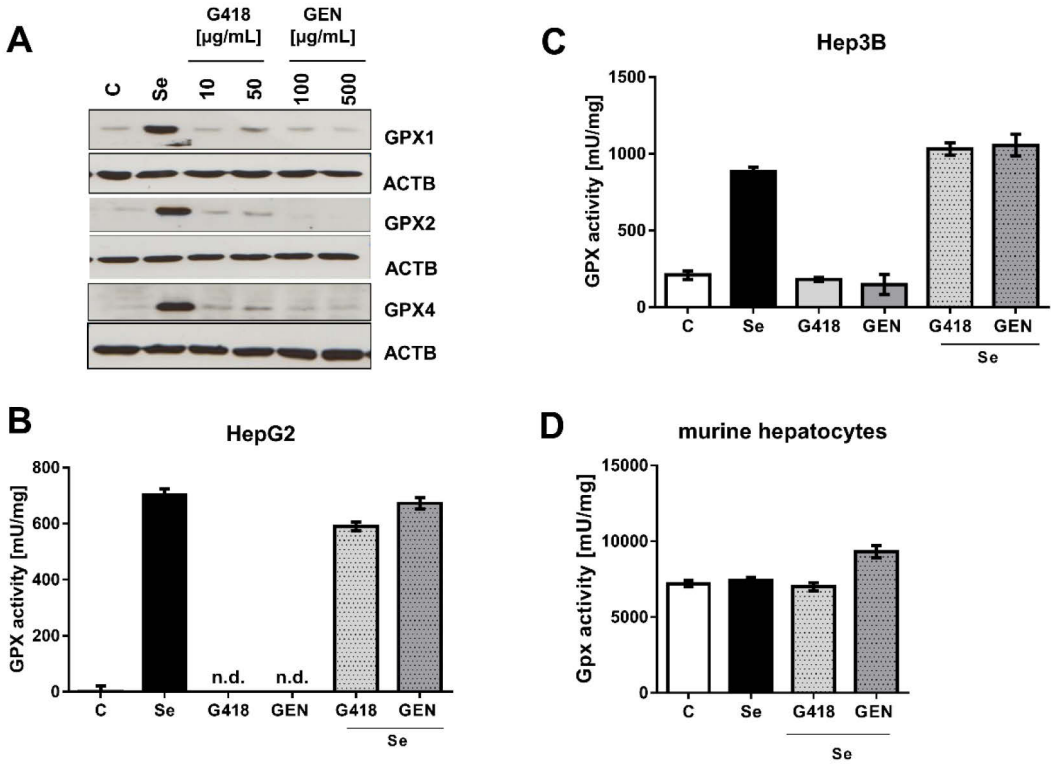


Figure 29: G418 and gentamicin affect the GPX expression.

Se-supplemented and Se-depleted HepG2, Hep3B and primary murine hepatocyte cells were stimulated with G418 (10 µg/mL or 50 µg/mL) and gentamicin (100 µg/mL or 500 µg/mL) for 48 hours. (A) Western blot analysis of HepG2 cells demonstrate that 50 µg/mL G418 induced GPX protein expression, but remained markedly below the Se-control. A slightly greater induction of GPX1 and GPX2, compared to GPX4 was observed when stimulated with 50 µg/mL G418. Gentamicin had no effect. (B) In Se-depleted HepG2 cells, no GPX enzyme activity was detectable when treated with G418 or gentamicin alone. A combined stimulation with Se resulted in a comparable increase when compared to the Se-control. Similar effects were observed for (C) Hep3B cells and (D) primary murine hepatocytes (Mean ± SEM, n=3-4).

Similar results were observed in Hep3B cells (Figure 29 C) and primary murine hepatocytes (Figure 29 D). It is noteworthy that the GPX enzyme activity in Se-supplemented hepatocytes was slightly higher when stimulated with gentamicin rather than G418.

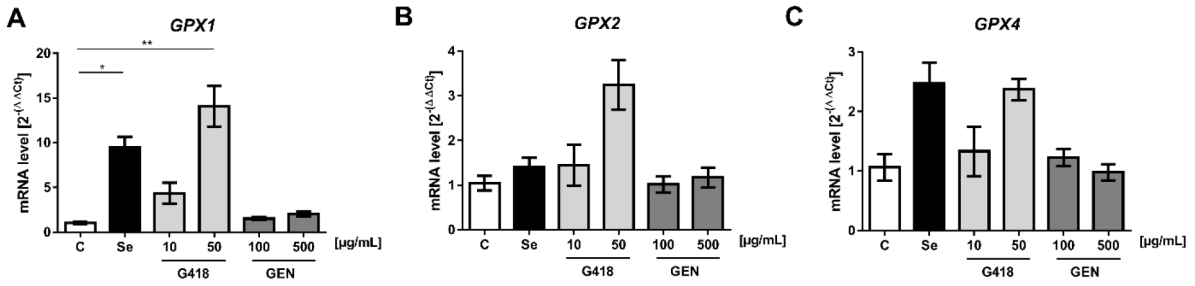


Figure 30: AG affect the GPX transcript levels in HepG2 cells.

HepG2 cells were stimulated for 24 hours with Se, G418 (10 µg/mL or 50 µg/mL) or gentamicin (100 µg/mL or 500 µg/mL). The transcript levels of *GPX1*, *GPX2* and *GPX4* were determined by quantitative RT-PCR. (A) Se and 50 µg/mL G418 strongly induced the *GPX1* transcript level. Only minor effects were observed for gentamicin (1.5-fold and 2.0-fold, respectively). (B) A marked increase in the *GPX*2 mRNA expression by 50 µg/mL G418, minor effects by 10 µg/mL G418 and Se, but no effects by gentamicin were noted. (C) Higher concentrations of G418 induced the *GPX4* transcript level in a similar manner to Se alone (Mean ± SEM, n=4).

The relationship between AG and the transcript levels of *GPX1*, *GPX2* and *GPX4* was investigated (Figure 30). The AG-mediated induction of the transcript levels varied markedly between GPX isozymes. The Se-sensitive *GPX1* exhibited the strongest increase in transcript level (9.5-fold above control). Increasing the concentrations of G418 and gentamicin, a dose-dependent relationship between the *GPX1* transcript and G418, and to a comparatively minor extent gentamicin was observed (1.5-fold and 2.0-fold above control, respectively) (Figure 30 A). It is worth highlighting that 50 µg/mL G418 induced the transcript level of *GPX1* to a greater extent than Se alone (14.0-fold to 9.5-fold). In comparison, a 3.0-fold induction in the *GPX2* transcript level was reached in cells treated with 50 µg/mL G418. The effect of Se on the *GPX2* transcript level was negligible (Figure 30 B). Higher concentrations of G418 induced highly Se-prioritised *GPX4* transcript levels similar to Se (Figure 30 C). Collectively, lower concentrations of G418 and gentamicin had only negligible effects on *GPX2* and *GPX4* transcripts.

In order to investigate the effects of AG on the expression and enzymatic activity of DIO1, HepG2, Hep3B and primary murine hepatocyte cells were stimulated with G418 (10 or 50 µg/mL), gentamicin (100 or 500 µg/mL) and Se for 48 hours. Following stimulation, the transcriptional and translational levels of DIO1 and the enzyme activity were quantified. The results show that supplemental Se is fundamental for the basal DIO1 expression (Figure 31 A). The DIO1 enzyme activity when stimulated with G418 or gentamicin alone remained under limit for detection by the assay (Figure 31 A, background threshold is indicated as the lower dashed line), whereas Se-supplemented cells strongly promoted the DIO1 enzyme activity. A combined application of Se with either G418 or gentamicin, did not alter the Se-dependent DIO1 enzyme activity. It is interesting to mention that DIO1 enzyme activity decreases at higher AG concentrations.

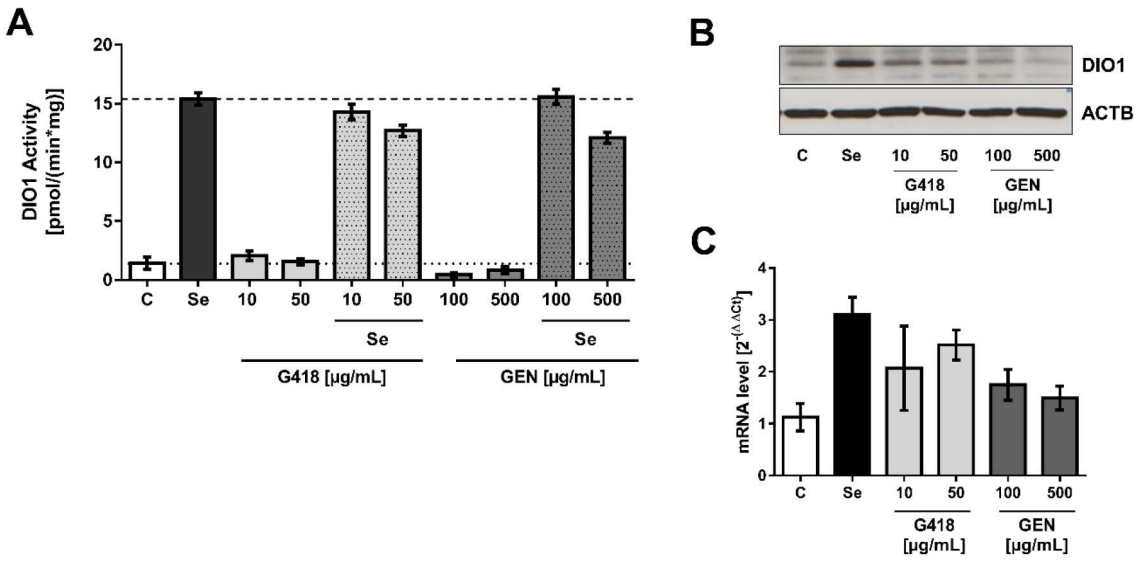


Figure 31: Effects of G418 and gentamicin on DIO1 expression.

Se-supplemented and Se-depleted HepG2 cells were stimulated with G418 (10 µg/mL or 50 µg/mL) and gentamicin (100 µg/mL or 500 µg/mL) for either 24 hour or 48 hours, and the effects on the DIO1 expression and enzyme activity were analysed. (A) A sufficient Se-supplementation is mandatory for a basal DIO1 enzyme activity. No enzymatic activity above the background threshold (indicated as lower dashed line) was observed in cells stimulated with G418 or gentamicin alone. A combined stimulation with Se and G418 or gentamicin showed similar enzyme activity levels as when stimulated with Se alone. A slight decrease in the DIO1 enzyme activity was observed at higher AG concentrations. (B) Western blot analysis showed an induction of DIO1 protein expression when treated with G418, but not in response to gentamicin. (C) The transcript level of *DIO1* was 3-fold upregulated by Se, and to a lesser extent by G418 and even less by gentamicin (Mean ± SEM, n=4).

G418 marginally up-regulated the DIO1 translation (Figure 31 B) and transcription (Figure 31 C) levels, while gentamicin-mediated effects were negligible. In order to determine whether up-regulation of DIO1 expression occurs pre- or post-transcriptional, the translational regulation of DIO1 was investigated in HEK293 cells. These HEK293 cells are stably transfected with an artificial *DIO1* expression construct. The *DIO1* expression construct contains the cDNA originated from human *DIO1* gene, as well as a FlagTag allowing specific targeting by anti-Flag antibodies [Stoedter, et al., 2015]. This construct allows studies on post-transcriptional regulations independent of promoter- or transcriptional regulation.

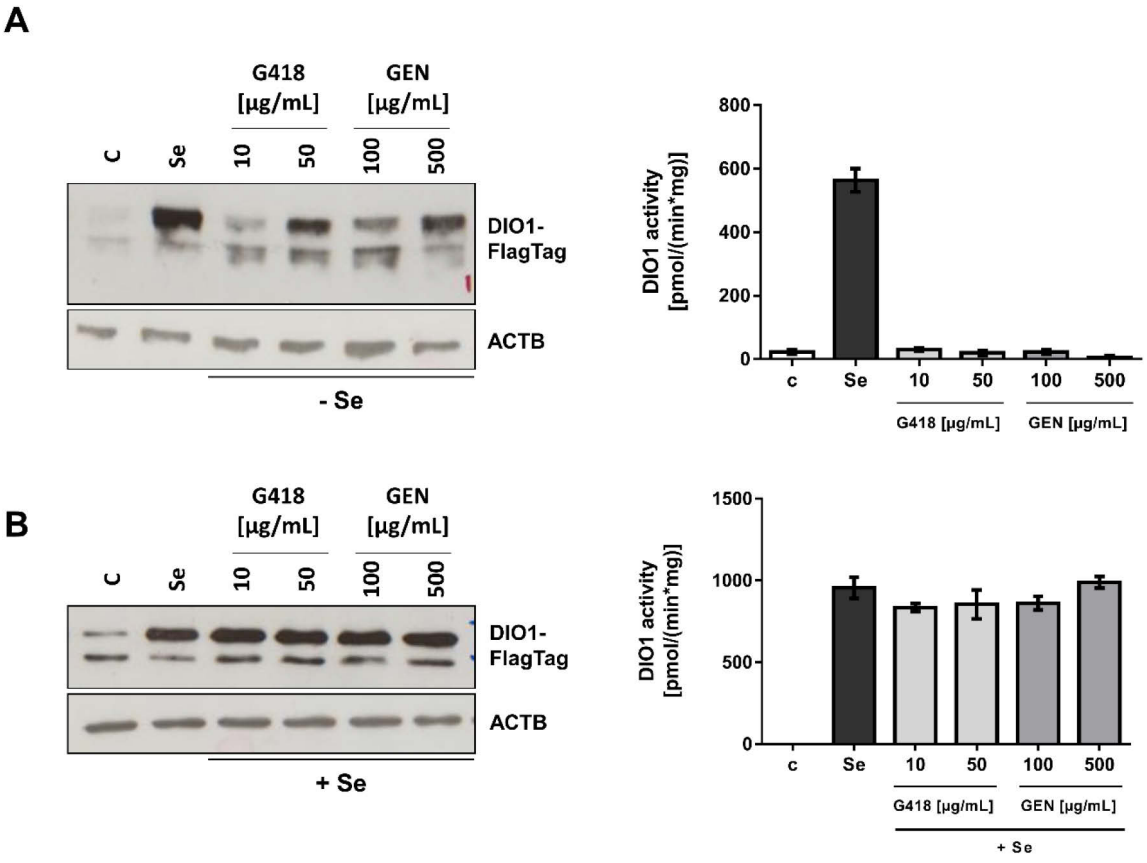


Figure 32: AG affect the DIO1 expression on post-transcriptional level.

Stably transfected HEK293 cells, containing a *DIO1* expression construct, were stimulated with G418 and gentamicin in the presence or absence of Se for 48 hours. The *DIO1* expression construct allows studies on post-transcriptional regulations independent of promoter- or transcriptional regulation (A) The DIO1 protein expression was strongly induced by both, G418 and gentamicin in a concentration-dependent manner as compared to the negative control. However, the DIO1 enzyme activity in Se-depleted cells was under the detection limit of the assay (background threshold indicated as lower the dashed line). (B) Supplemental Se strongly increased the DIO1 protein expression, while no changes in the enzymatic activity were observed when co-stimulated with G418 or gentamicin (Mean ± Se, n=6).

Se-supplemented and Se-depleted HEK293 cells containing the *DIO1* expression construct were stimulated with G418 or gentamicin, and the DIO1 protein and enzyme activity level were examined (Figure 32). The DIO1 protein level was strongly induced by G418 and gentamicin in a concentration-dependent manner, but to a lesser extent in comparison to the Se-control. In line with the endogenous DIO1 enzyme activity, the DIO1 enzyme activity in Se-depleted HEK293 cells remained under the detection limit of the assay (Figure 32 A, background threshold indicated as lower dashed line). While supplemental Se strongly promoted DIO1 protein expression (Figure 32 B), Se-supplementation in combination with G418 or gentamicin had no additive or modulating effect on the DIO1 expression level. Collectively, these data show that AG induce the biosynthesis of Se-free DIO1 along with the Se-induced DIO1 synthesis resulting in the co-existence of Se-containing and Se-free DIO1.

3.4. Synergistic effects of IL-6 and AG on SEPP biosynthesis

The previous sections have only addressed the effects of IL-6 and AG (G418 and gentamicin) on the expression of SEPP separately. As AG are applied to critically infected patients, i.e. characterised by high IL-6 serum levels [Wiehe, et al., 2016], we wanted to test in a final step the synergistic effects between both stimuli on SEPP expression. For this reason, the impact of G418 or gentamicin, in combination with IL-6 on SEPP expression in HepG2 cells was investigated. Se-supplemented and Se-depleted HepG2 cells were stimulated with G418 or gentamicin in combination with IL-6 for 48 hours and changes in the SEPP expression were analysed by Western blot and Enzyme-linked Immunosorbent assay (Figure 33).

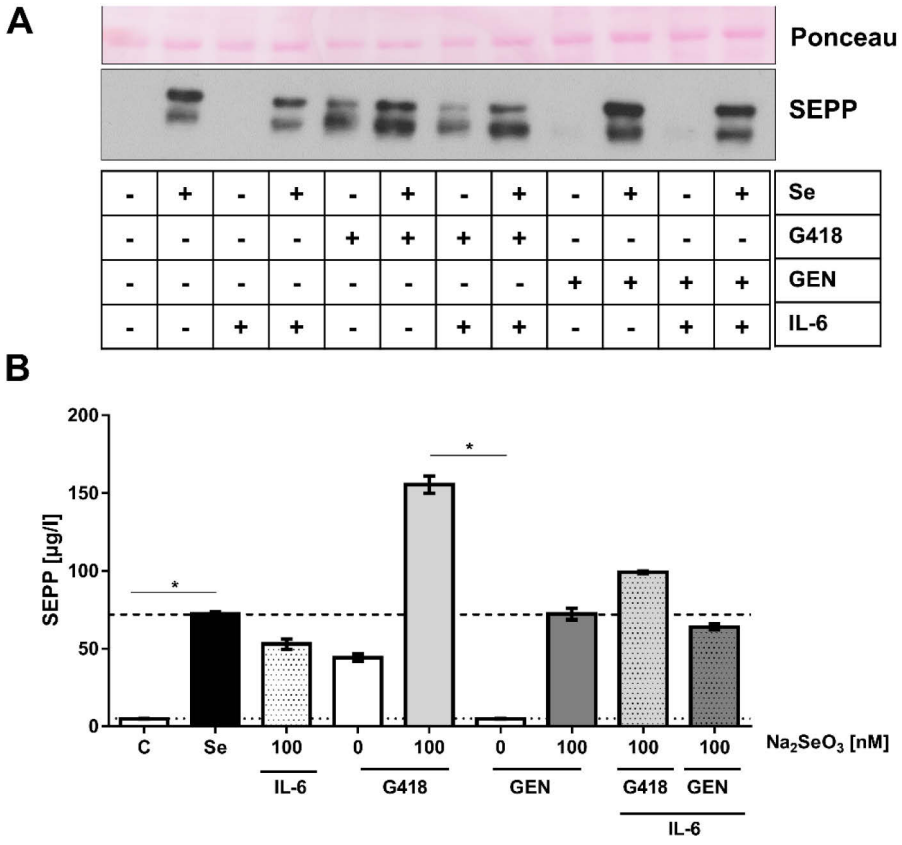


Figure 33: Synergistic effect of IL-6 and AG on SEPP expression in HepG2 cells.

Se-supplemented and Se-depleted HepG2 cells were stimulated with 50 µg/mL G418 or 500 µg/mL gentamicin in a combination with IL-6 for 48 hours. Changes in the SEPP expression in response to these stimuli were then analysed by Western blot analysis and Enzyme-linked Immunosorbent assay. IL-6 decreased SEPP expression, while G418 and but not gentamicin strongly increased the expression of SEPP. A combined stimulation of IL-6 and either G418 or gentamicin diminished the IL-6-mediated SEPP repression as observed in (A) western blot analysis and (B) Enzyme-linked Immunosorbent assay (Mean ± SEM, n=3).

Consistently, the SEPP Western blot analysis (Figure 33 A) and SEPP-ELISA data (Figure 33 B) demonstrate that IL-6 down-regulated the expression of SEPP, while G418 strongly up-regulated SEPP expression. A combined application of Se and G418 resulted in a 2.0-fold higher SEPP biosynthesis than supplemental Se alone. A combined stimulation with IL-6 and either G418 or gentamicin partially diminished the IL-6-induced decrease in SEPP expression.

G418 has proven to promote the biosynthesis of Se-depleted SEPP, but equivalent data regarding the Se-load per SEPP molecule under IL-6 treatment remain unknown. Focusing on the interplay of G418 and IL-6 in respect to SEPP expression, we wanted to determine whether the SEPP synthesised in the presence of IL-6 might also be Se-deficient, or whether the IL-6-mediated deceleration in SEPP biosynthesis may elevate the Se-incorporation into the SEPP. For this reason, HepG2 cells were treated with IL-6, G418 and/or Se for 48 hours and the molar ratio of Se per SEPP was determined as described previously (Figure 34).

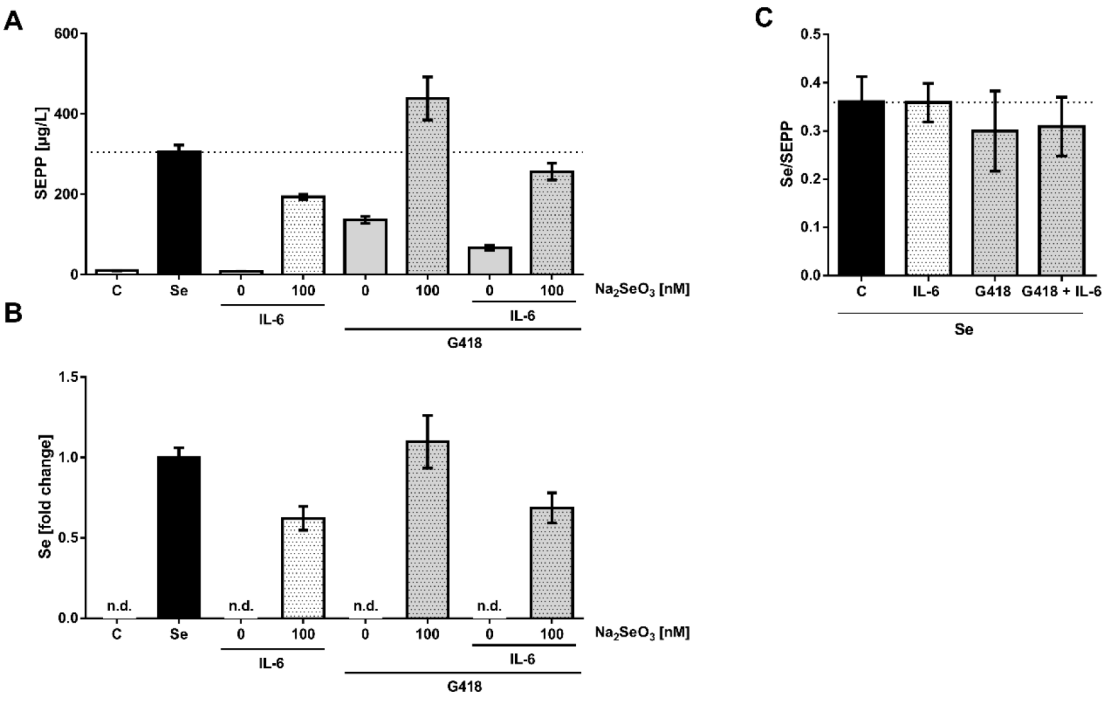


Figure 34: Effects of IL-6 and G418 application on the Se-load of SEPP.

HepG2 cells stimulated with IL-6 in combination with G418 and/or Se for 48 hours. The SEPP and Se-levels were measured in the conditioned media. (A) Supplemental Se and G418 strongly induced the SEPP biosynthesis in HepG2 cells. A combined application of Se and G418 resulted in an additive increased SEPP expression, whereas an additional IL-6 treatment reduced the SEPP concentration. (B) In parallel, no Se was detected in the immobilised proteins of Se-depleted cells. A co-simulation with sodium selenite led to an increase in Se-level in both IL-6 and G418-treated cells. However, the Se-concentration was lower in IL-6 stimulated cells. (C) The molar ratio of Se per SEPP showed that Se was only detectable in immobilised SEPP when additionally supplemented Se. No differences in the Se-load were found in cells treated with IL-6, whereas a combination with G418 reduced the Se-load per SEPP molecule (Mean ± SEM, n=3).

In agreement with previous results (Figure 33), we found that G418 induced the SEPP biosynthesis in Se-depleted HepG2 cells, although to a lower extent than Se alone (Figure 34 A). This synthesised SEPP appeared to be Se-deficient (Figure 34 B). A combined application of Se and G418 resulted in an additive increase in the SEPP concentration as described in earlier findings. However, the ratio of Se per SEPP revealed a slightly lower Se-load per SEPP molecule in G418-treated cells. When cells were treated with IL-6 and G418, the SEPP and Se-levels decreased in comparison to their according control stimuli. Interestingly, the drop in the Se- and SEPP-levels were marginally lower in the presence of G418. IL-6 decreased the SEPP expression, but did not affect the Se-load per SEPP regardless of an additional G418 application (Figure 34 C).

Taken together, IL-6 and G418 affect the SEPP expression in a different direction and through different molecular mechanisms. While IL-6 down-regulates SEPP expression, G418 leads to an increased biosynthesis of Se-deficient SEPP isoform and simultaneously decreased Se-loaded SEPP isoform. These different modes of SEPP dysregulation by IL-6 and G418 lead to a more exaggerate negative effect of SEPP.

3.5. Summary of results

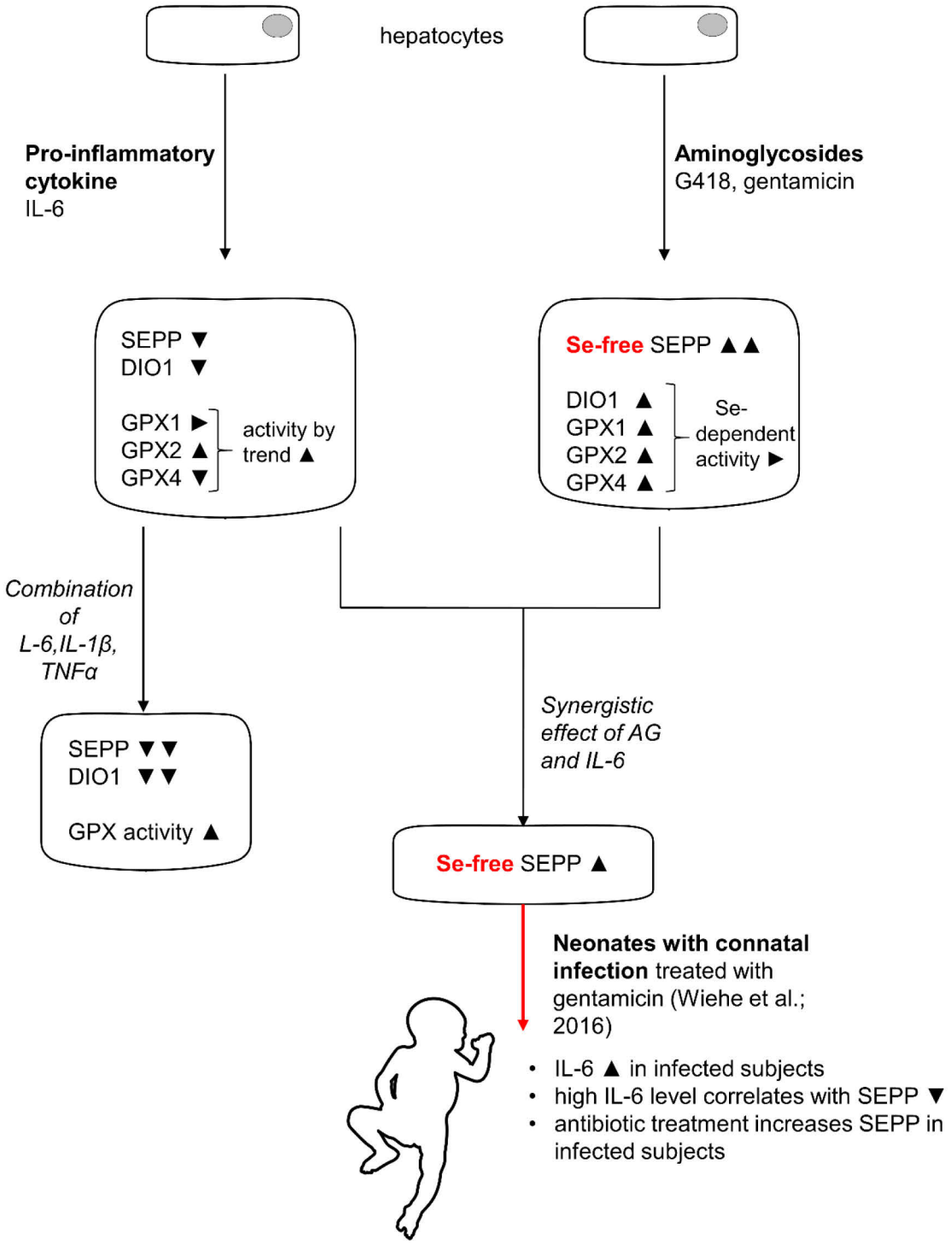


Figure 35: Graphical summary of the main results.

▲ increased, ▲▲ strongly increased, ►unaffected, ▼decreased, ▼▼ strongly decreased

4. Discussion

Se and selenoproteins play an important role in inflammation and immunity by regulating the reactive oxygen species and redox status. Some clinical trials have shown that Se and SEPP decrease in sepsis and negatively correlate with the severity of the disease [Angstwurm, et al., 2007; Forceville, et al., 1998]. Other studies have concluded that supplemental Se improves the survival rate and leads to a more rapid improvement of organ dysfunction during sepsis [Angstwurm, et al., 2007]. In a study of neonates with connatal infections, the Se and SEPP plasma concentrations correlated inversely with IL-6 and Se positively with increasing gentamicin concentrations, while the plasma SEPP concentrations increased after gentamicin application [Wiehe, et al., 2016]. In a similar study of neonatal children with proven and clinical sepsis the plasma SEPP concentration dropped to a greater extent than plasma Se-concentrations, where the IL-6 plasma concentration increased in parallel [Asci, et al., 2015].

This work delivers insights into the molecular interplay between pro-inflammatory cytokines in particularly IL-6, aminoglycoside antibiotics, the Se-status and their synergistic effects on the expression of hepatic selenoproteins during critical illness and attempts to explain why the plasma Se- and SEPP-concentrations drop during sepsis.

4.1. Pro-inflammatory cytokines redirect hepatic selenoprotein expression

4.1.1. Pro-inflammatory cytokines reduce SEPP expression

The acute phase response (APR) is an orchestrated response by the immune system against infection, inflammation or tissue injury, involving the elevated expression of different pro-inflammatory cytokines including IL-6, IL-1β and TNFα. Although all three cytokines increase during the APR, IL-6 is usually detectable in high concentrations in serum during illness and acts as a systemic hormone [Boelen, et al., 1993]. These cytokines act as mediators of the APR and promote secretion of acute phase proteins by the liver [Moshage, 1997]. Liver-derived acute phase proteins include hepcidin, C-reactive protein, procalcitonin or ceruloplasmin (CP). Hepcidin, which is directly regulated by IL-6, contributes to the redistribution of iron (Fe) away from the circulation into intracellular storage sites and thereby reducing the availability of this essential trace element to the invading pathogens while supporting endogenous haemoglobin synthesis [Darveau, et al., 2004; Nemeth, et al., 2004].

The copper-transporter CP is another IL-6 regulated acute phase protein and shows increasing plasma concentrations under inflammatory conditions [Goldstein, et al., 1982] including sepsis [Chiarla, et al., 2008]. During the acute phase response, the intracellular copper levels decrease and the hepatocyte CP secretion increases. High copper concentrations are noted for their bactericidal properties. CP is capable of oxidizing toxic ferrous to the more stable ferric ions and thereby reducing ROS concentrations [Roeser, et al., 1970].

Three IL-6 response elements have been identified in the CP gene [Conley, et al., 2005]. Intracellular IL-6 signalling classically occurs via the JAK/STAT signalling pathway and involves the translocation and binding of the dimerised STAT3 transcription factor to its response elements within the promoter of its target gene. However, subsequent studies of the CP promoter have shown that the IL-6-incuded CP expression involves the Forkhead box protein O1 (FOXO1) rather than STAT3 [Sidhu, et al., 2011]. FOXO1 is a transcription factor linked to the antioxidative response [Klotz, et al., 2015]. In parallel, an upregulation of SEPP via FOXO1 has been described [You, et al., 1994]. SEPP comprises both antioxidative and peroxynitrite-scavenging properties [Arteel, et al., 1998]. Beyond the antioxidative role, previous studies have demonstrated that for inflammation SEPP qualifies as a negative acute phase protein [Hollenbach, et al., 2008] and can act as the most sensitive biomarker of Se-status in septic shock [Forceville, et al., 2009]. In this context, our data regarding an IL-6 mediated regulation of SEPP clearly support this contrary regulation of CP and SEPP. In line with this, the Western blot analysis and ELISA-data collectively show that IL-6 decreases the secretion of SEPP by hepatocytes as shown for HepG2 and Hep3B cells (Figure 11). However, in this study it has been shown that the drop in secreted SEPP is not the result of reduced secretion, but rather caused by a direct down-regulation of SEPP biosynthesis as indicated by reduced *SEPP* transcription (Figure 11) and promoter activity (Figure 12). This result is in line with a recent clinical study highlighting a significant association between two SNPs (rs72554691; rs7719242) in *SEPP* with IL-6 levels [Hellwege, et al., 2014].

Pilot studies of the human *SEPP* promoter suggest that the IL-6-mediated repression might occur via a response element within the proximal or core promoter region of the gene (Figure 13). The strength of repression and the proximity to the transcription start imply that SEPP is a direct and preferred target of IL-6 signalling. However, this experimental design only allows a constriction of the regulatory element and further studies employing methods such as chromatin immunoprecipitation sequencing (ChiP-Seq) could be better suited to identify distinct IL-6 response element sequences.

Repressive response elements in the human *SEPP* promoter have also been identified for the pro-inflammatory cytokines Interferon-γ (IFN-γ), IL-1β, TNFα [Dreher, et al., 1997] and the anti-

inflammatory cytokine transforming growth factor beta (TGFβ) [Mostert, et al., 2001]. While IFN-γ, IL-1β and TNFα promote hepatic acute phase proteins [Gruys, et al., 2005], TGFβ is linked to the promotion of hepatic fibrosis and carcinogenesis [Yang, et al., 2013]. In this respect, the interplay the pro-inflammatory cytokines IL-6, IL-1β and TNFα on the SEPP expression were studied. While an isolated application of IL-1β or TNFα slightly induced SEPP secretion by HepG2 cells, a single or combined IL-6 treatment strongly reduced the biosynthesis of SEPP (Figure 18). More importantly, a combination of IL-6 with either or both cytokines suppressed SEPP expression even more greatly than IL-6 alone. Similarly, SEPP expression was found to be reduced by IL-6 in Hep3B cells in combination with other pro-inflammatory cytokines. These findings are in agreement with a similar study by *Speckmann et al*. highlighting that the individual cytokines IL-1β, IFN-γ and TNFα reduce protein and transcript levels of SEPP to a minor extent, whereas a combination of these cytokines caused an over 50% decrease in the SEPP expression in colorectal adenocarcinoma cells Caco-2 [Speckmann, et al., 2010]. Taken together these findings indicate that SEPP is a vulnerable target of depression by different pro- and anti-inflammatory cytokines and thereby highlights the importance of improvements to Se-metabolism during inflammatory processes.

The liver is the central organ in systemic Se metabolism and a drop in SEPP expression leads onto negative effects on the peripheral Se-supply and leads to Se-deficiency of SEPP target tissues. The question arises how does the organism selectively down-regulates Se-availability to prioritise the biological use of Se. It can be assumed that the livers response to IL-6 is to increase intracellular Se-concentrations when hepatic SEPP expression decreases in order to redistribute Se and thus boosting the production of intracellular selenoproteins as observed in Sepp knockout mice. In these knockout mice, Se-concentrations increased especially in liver, but declined in serum and other organs [Schomburg, et al., 2003].

4.1.2. Pro-inflammatory cytokines regulate selenoprotein expression

To test whether the assumption holds true that hepatic SEPP expression and secretion decreases in order to elevate the intracellular Se-concentrations and thereby boosting the production of intracellular selenoproteins in liver cells, we analysed the expression of various hepatic selenoproteins in response to IL-6.

In HepG2 cells, there was an overall significant increase in GPX enzyme activity and a subtle, but obvious trend in both Hep3B cells and primary murine hepatocytes (Figure 14). These finding are in accordance with a recently published study on the oxidant and antioxidant status in neonatal children with proven and clinical sepsis, where the erythrocyte GPX enzyme activity

was increased, along with IL-6 plasma concentration [Asci, et al., 2015]. However, a deeper view revealed an isozyme specific response to IL-6. The isozyme specificity is characterised by: the Se-sensitive *GPX1* remaining mainly unaffected by IL-6, *GPX4* showing a lower promoter activity (Figure 16) and significant transcript level (Figure 15). By contrast, GPX2 was strongly induced by IL-6, most likely via direct associations with promoter regulation. This association could be linked with a STAT-mediated induction of Gpx2 shown during acute colitis as described by *Hiller et al.* (2015). Co-localisation of Gpx2 and nuclear STAT3 in inflamed areas highlight the role of Gpx2 in inflammatory response [Hiller, et al., 2015]. In inflammation-triggered carcinogenesis, the induction of GPX2 leads to inhibition of oxidation-induced apoptosis and supports the self-renewal of affected intestinal mucosa [Parkkinen, 1989]. In *Gpx2* knockout mice, Gpx1 is able to partially compensate for the Gpx2-mediated inhibition of acute inflammation [Florian, et al., 2010]. In hepatocytes the up-regulation of GPX2 in response to IL-6 may be meaningful in the reduction of intracellular oxidative stress, which can account for tissue damage and further negative outcomes.

Despite these promising findings, it cannot be proven that the observed increase in GPX enzyme activity solely relies on the up-regulation of GPX2, and if the increase in GPX enzyme activity conclusively curtails increasing levels of intracellular ROS. On the one hand, further experiments with GPX isozyme-specific substrates, e.g. tert-butyl hydroperoxide or cumene hydroperoxide, are needed in order to identify the isozyme-specific responses to IL-6. On the other hand, IL-6 only acts as a mediator of the acute phase response, but it does not provide information about the actual ROS status. Thus experiments determining the intracellular ROS-level in relation to the individual GPX-isozymes could be achieved using the ROS indicator Dihydrorhodamine 123 [Yazdani, 2015].

This study has demonstrated that a co-application of the cytokines IL-6, IL-1β and TNFα resulted in an even higher GPX enzyme activity in HepG2 and Hep3B cells, and in a relatively subtle increase in primary murine hepatocytes. This observation provides evidence for an additive and thereby physiological effect. Although, the initial observations of the GPX protein expression levels did not explicitly support this hypothesis (Figure 19). Further studies are needed in order to better characterise this additive increase in GPX enzyme activity. Currently published but isolated studies on GPX expression in response to different cytokines point towards a complex picture. In human umbilical vein endothelial cells, IL-1β increased the expression level and enzyme activity of GPX4, and 1 ng/mL TNFα increased the GPX4 activity while 3 ng/mL TNFα reduced both the enzyme activity and transcript level [Sneddon, et al., 2003]. These dynamic regulatory changes at different cytokine concentrations might explain

why the plasma Se strongly decreases in critical ill patients and inversely correlates with the mortality rate [Angstwurm, et al., 2007].

A liver-specific inactivation of Dio1 activity in mice has illustrated that the hepatic deiodinase activity is dispensable for the maintenance of normal thyroid hormone levels [Streckfuss, et al., 2005]. The investigation of DIO1 in hepatic-derived cells illustrate a strong negative impact of IL-6 and display a direct repression of the *DIO1* promoter by IL-6 (Figure 17). These findings are in line with similar observations found in cell culture, rodent sepsis models and human patient tissues [Boelen, et al., 1996; Xu, et al., 2014]. In this context, the decrease in serum T3 during illness proved to be significantly related to serum IL-6 concentrations [Boelen, et al., 1996]. DIO1 catalyses the activation of the prohormone T4 to T3. A drop in the DIO1 enzyme activity in response to IL-6 most likely causes a decrease of serum T3 in human patients. These low T3 level can manifest as low-T3 syndrome, also known as Non-Thyroidal Illness Syndrome (NTIS). NTIS is found in seriously ill or cachectic patients characterised by low free T3, elevated rT3, normal or low Thyroid-stimulating hormone, and on long-term low free T4. NTIS appears in many patients of the intensive care unit and correlates with a poor prognosis in patients with critically low T4 level [DeGroot, 2000]. As DIO1 expression is low in most hepatic-derived cell lines that were utilised in our studies, the findings are limited to HepG2 cells at present. It would be of interest to study other IL-6 responsive cells to determine if effects on DIO1 are liver-specific or rather cause of a systemic down regulation of DIO1. The results of such an investigation may better explain the resulting effects on circulating thyroid hormone concentrations.

The application of IL-6 in combination with IL-1β or TNFα caused a stronger and additive-like repression of DIO1 expression and enzymatic activity. Preliminary studies revealed that DIO1 is repressed by various pro-inflammatory cytokines [Jakobs, et al., 2002; Xu, et al., 2014]. This strong repression of DIO1 in response to several pro-inflammatory cytokines might even worsen the clinical outcome of NTIS. Presently, our studies focused on IL-6-mediated effects on Se-replete cells, as hepatocytes do not experience immediate Se-deficiency in inflammatory diseases. This detail may explain the discordant findings in comparison to studies investigating serum-free, Se-deprived hepatocytes in culture [Martitz, et al., 2015; Wajner, et al., 2011].

4.2. AG interfere with selenoprotein biosynthesis

4.2.1. AG-induced UGA codon readthrough is selenoprotein-specific

Although the selenoprotein biosynthesis machinery is evolutionary well-conserved, different genotypes, sex- and age-specific effects [Donovan and Copeland, 2009], or changes in hormone [Bubenik and Driscoll, 2007] or cytokine level have proven to modulate the selenoprotein biosynthesis [Martitz, et al., 2015]. It is clinically necessary that certain drugs, e.g. statins and metformin are able to alter the biosynthesis of selenoproteins [Moosmann and Behl, 2004; Speckmann, et al., 2010; Speckmann, et al., 2009]. It is therefore important to characterise pharmacological agents that might have either a beneficial or a harmful impact on the expression of selenoproteins. This is particularly important for pharmaceutical agents that are utilised for patients with critical infectious diseases where Se- and SEPP concentrations are already negatively affected.

With the previous statement in mind, the class of aminoglycoside antibiotics have moved into the focus of selenoprotein biosynthesis and critical illness research. Briefly, AG promote the misinterpretation of all UGA codons, including those found in the selenoprotein-coding open reading frame. Studies on the impact of AG on selenoprotein expression are limited. However, these few studies have already discovered a complex, rather than straightforward process that leads to interference with selenoprotein biosynthesis. It is this complex process we have aimed to unravel, particularly identifying important features that are involved in this puzzling network. We designed a dual luciferase-based reporter construct consisting of a fusion protein of FLuc and RLuc luciferase interrupted by a UGA stop codon and a selenoprotein specific SECIS-element in the untranslated 3'UTR region (Figure 10).

Various luciferase-based reporter constructs have been generated based on the different characteristics of selenoproteins, i.e. the Se-sensitive *GPX1*, the high-hierarchic *GPX4* and *SEPP* which is the only selenoprotein gene containing two SECIS-elements and more than one Sec-residue, and a SECIS-free reporter that allows SECIS-independent studies of the UGA codon. The data presented in this study have illustrated fundamental differences in the reporter gene activities with respect to the Se-status (e.g. supplemented or deficient). The *GPX1*-originated SECIS-element exhibited a more steady response to Se when compared to the *GPX4*-derived SECIS-element (Figure 21). These data are in agreement with a similar study in HEK293 cells investigating the differential expression of *GPX1* and *GPX4* in relation to their SECIS-elements [Latreche, et al., 2012]. Unlike our reporter constructs, the UGA was directly cloned into the luciferase open reading frame, which bears the disadvantage when controlling and standardising transfection efficiencies. This study highlighted that the UGA

readthrough efficiency strongly depends on the nature of the SECIS-element, but also of the translation factors EFsec and SBP2 [Latreche, et al., 2012].

With respect to the two SECIS-elements found in the human *SEPP* gene, we also found great differences in their Se-response. While the SECIS1-element was Se-responsive, a tandem of both SECIS-elements (SECIS1&2) showed only a gradual response to Se, even at higher Se-concentrations. These findings are in agreement with a detailed study on the different functions of the two SEPP SECIS-elements and the different efficiency of UGA recoding. While SECIS1 is essentially required for the biosynthesis of full-length *SEPP*, SECIS2 is needed for recoding the first UGA in the *SEPP* transcript. SECIS2 functions as a control point for synthesis requiring the presence of additional factors (e.g. Sec) and thus is key to SEPP translation [Stoytcheva, et al., 2006]. This function may rationalise the low UGA recoding efficiency observed for SECIS2. Collectively, these studies provide some explanation of the mechanism affecting the hierarchy of selenoproteins [Martitz, et al., 2015; Schomburg and Schweizer, 2009].

We have screened several AG (streptomycin, amikacin, tobramycin, gentamicin and neomycin) for possible UGA readthrough (Figure 22) independent of the SECIS-element. Interestingly, only G418 and gentamicin were able to induce a significant UGA readthrough, while the effect of the other AG were negligible. Although all AG inhibit the protein biosynthesis via interfering with the small ribosomal subunit, the effect on the UGA readthrough differs markedly. In this context, a comparative analysis of the effect of AG on bacterial protein synthesis *in vitro* revealed great differences in their protein inhibition response [Zierhut, et al., 1979]. Accordingly, AG are clustered into three groups. Firstly, AG that inhibit the protein biosynthesis in a monophasic way potentially via one single inhibitory site. Examples for this include streptomycin and hygromycin B. Secondly, AG that inhibit the protein synthesis in a triphasic way indicating a multiple interaction of AG and ribosome. Gentamicin, neomycin and tobramycin belong to this category. Interestingly, these AG promote the synthesis of prolonged proteins rather than early termination. Lastly, AG such as garamine and lividamine respond in a biphasic way [Zierhut, et al., 1979]. The different AG excretion rates, AG uptake rates, toxicity levels and affinity to the ribosome are additional factors explaining the great differences in their effects on the UGA readthrough efficiency. Collectively, these aspects might explain the observed differences in the UGA readthrough by different AG.

Currently published studies that investigated effects of AG on selenoprotein expression have mainly focused on geneticin (G418). However, G418 is not regularly used in the clinics, but is structural similar to the clinically applied gentamicin. Our data collectively demonstrate that G418 supports a several-fold higher UGA readthrough than gentamicin (Figure 23). These findings are in agreement with previous studies comparing different AG in different reporter

systems where G418 but not gentamicin, tobramycin or amikacin were able to strongly induce the translational readthrough of the stop codon (UAG A) as studied in therapeutic strategies of proximal spinal muscular atrophy [Heier and DiDonato, 2009].

On average, the G418-sensitivity of the different SECIS-reporters was qualitatively very similar to their Se-response (Figure 23). These findings highlight that both processes may interact with each other, by either direct interference, synergism or neutralisation. The nature of this interaction was tested by a combined application of Se and G418. We found that the *GPX1*-derived SECIS reporter showed a similar response to supplemental Se when compared to a combination of Se and G418 (Figure 24). By contrast, the *GPX4*-originated reporter construct exhibited a synergistic maximal expression upon stimulation with the combination of Se and G418. These different findings highlight the importance of the SECIS-element for the interaction and imply that different selenoprotein transcripts are not equally affected by AG and the actual Se-status. As the SECIS-element sequences of the 25 human selenoprotein genes share only few sequence similarities, it will be mandatory to compare their specific sensitivities to AG-mediated readthrough side by side, and then to predict the modulating effects of Se-supplementation on this interference in order to extrapolate potential effects on their relative biosynthesis in patients under AG treatment.

Although a general effect of AG on biosynthesis of selenoproteins has been shown before [Gupta and Copeland, 2007; Handy, et al., 2006; Tobe, et al., 2013], there is little information about the interplay of the AG with other selenoprotein-specific factors [Martitz, et al., 2016]. In this respect, our findings revealed that the UGA recoding efficiency depends on the chosen AG, the particular SECIS-element and the actual Se-status. As our results were obtained in human cells, the data complete similar findings of *Grupta et al.* who revealed the interplay between translation termination, Sec codon context, and *Sec-insertion sequence-binding protein 2 (*SBP2*)* in a rabbit reticulocyte system [Gupta and Copeland, 2007]. The findings are also in agreement with *Handy et al.* showing the differences in the response of Se-depleted vs. Se-supplemented COS7 cells to G418-induced readthrough [Handy, et al., 2006]. Notably, the data complement earlier findings towards a clear dependence upon the individual SECIS-element.

Despite these findings, our studies are limited in some respects a) only a small number of available AG were investigated and b) only a group of four different SECIS-elements were studied. However, the reductive character of this system is advantageous to understand the molecular interplay of endogenous sequence-specific factors with translation-modulating activities of pharmacological interventions. For these reasons, the reporter system proves to be well suited in order to a) identify and characterise selenoprotein disruptors [Martitz, et al.,

2016] or b) quantify bioactive selenocompounds in human serum samples as described in a similar study on the Bone morphogenic protein (BMP) responsive luciferase based reporter, which allows quantification of BMP concentrations in human serum [Herrera and Inman, 2009].

4.2.2. AG affect the biosynthesis of hepatic selenoproteins

AG induce biosynthesis of Se-deficient SEPP in the liver

As SEPP is the only selenoprotein that comprises ten UGA codons allowing the incorporation of up to ten Sec-residues, we hypothesised that SEPP might be a sensitive target of AG-mediated UGA readthrough. In contrast to other selenoproteins, Sec-residues in SEPP are structural components fulfilling the transport function and a single exchange of Sec with other amino acids would not *per se* disrupt, but probably diminish, its overall function. Recently published research has shown that in healthy subjects a subset of SEPP molecules with reduced Sec-content did not result from truncation of the actual protein, but arose by insertion of amino acids alternative to Sec [Turanov, et al., 2015]. It is important to note that the Sec-incorporation into SEPP strongly relies on the Se-availability and will decrease, if Se becomes limited [Meplan, et al., 2009]. As the biosynthesis of SEPP already seems to include a reduced accuracy regarding the incorporation of Sec, the question emerged whether this might be further augmented by AG application.

The incubation of Se-depleted hepatoma cell lines with G418 had an enormous and almost equal inductive effect on SEPP biosynthesis when compared to a saturating Se-supplementation (Figure 25). Gentamicin, on the other hand, had a weaker, but still detectable positive effect on the SEPP biosynthesis, as found in Western blot analysis (Figure 25). It is worthy to mention that the inductive effect also was found in several cell types: human HepG2 and Hep3B cells and murine Hepa1-6 cells. This evidence argues for a species independent effect of AG on SEPP biosynthesis. These species independent findings are further bolstered by similar results of earlier studies investigating the effects of AG on the UGA readthrough efficiency in human-, monkey- and rabbit-derived cell systems [Gupta and Copeland, 2007; Handy, et al., 2006; Martitz, et al., 2016]. In line with the Western blot analysis, G418 at a concentration of 50 µg/mL was able to promote the *SEPP* transcript level to a similar extent when compared to supplemental Se (Figure 27).

In line with Western blot analysis, quantitation of SEPP revealed an increase of SEPP biosynthesis in G418-treated cells, to a similar extent of SEPP-concentrations obtained after Se-supplementation (Figure 26). These results suggest an AG-driven production of Se-free SEPP. In order to test this assumption, the Se-content per SEPP molecule derived from Se- and/or G418-supplemented cells was determined (Figure 28). It was found that the G418-derived SEPP from Se-depleted HepG2 cells was Se-free, i.e. the amount of bound Se was below the detection limit of this method. Notably an additional supplementation with increasing concentration of sodium selenite led to an increase in Se-content per SEPP and to a further increase of total SEPP. These findings point to an additive, inductive effect on the SEPP biosynthesis rather than a competition between Se and G418. It is considerable that sufficient supplementation with sodium selenite cannot revert the G418-induced Se-depletion in SEPP. However, this Se-supplementation leads to a change in the ratio of Se-depleted to Se-loaded SEPP in favour of the Se-loaded isoform, and thereby fulfilling its function as Se-transporter. These findings of G418-induced Se-depleted SEPP are in accordance with recently published results on gentamicin-treated neonates, where a correlation was found between gentamicin-levels and Se-levels, but not serum SEPP levels, which also points in the direction of alternations to Se-load of the SEPP-bound Se-fraction [Wiehe, et al., 2016].

During instances of critical illness, where SEPP serum concentration is low, treatment with AG antibiotics would further aggravate the situation by interfering with the physiological Se-distribution system. This becomes even more relevant when patients are chronically treated with AG, for example in cystic fibroses. In these patients the cystic fibrosis transmembrane conductance regulator (CFTR) gene contains a mutation introducing a premature termination signal that subsequently causes a deficiency or absence of functional chloride-channel activity. Under these circumstances, gentamicin treatment can cause a readthrough of this premature termination signal resulting in the full-length CFTR protein and thus correct the electrophysiological abnormalities caused by CFTR dysfunction [Wilschanski, et al., 2003].

AG promote the biosynthesis of non-functional GPX and DIO1

As AG have shown to strongly induce the biosynthesis of Se-depleted SEPP in hepatic-derived cells, the question arose; to what extent AG modulate the expression of selenoproteins, e.g. GPX and DIO1, which incorporate only one Sec-residue and might be therefore less sensitive to AG. Compared to the strong induction in SEPP biosynthesis by AG, the induction of GPX1, GPX2 and GPX4 biosynthesis was of a lesser extent (Figure 29). On average, lower concentrations of G418 and gentamicin had negligible effects on protein expression level of these GPX isozymes, while higher G418 concentrations moderately induced the protein expression levels. Furthermore, it appears that G418 induced the GPX biosynthesis in an isozyme-specific manner, where GPX1 was slightly stronger induced than GPX2 or GPX4. These findings are in agreement with the results from the SECIS-reporter studies where the *GPX1*-originated SECIS-element exhibited a more robust response to Se and G418 when compared to the *GPX4*-derived SECIS-element. These apparent isozyme-specific differences were also found at GPX transcript levels, where *GPX1* showed an exceptionally high induction in transcript level in response to G418 (~15fold), even above the effect observed upon sodium selenite stimulation (~10fold). By contrast, stimulation with Se and G418 induced the *GPX4* transcript level to a smaller degree. Interestingly, the *GPX2* transcript level was unaffected by supplemental Se, but increased in response to G418. Collectively, these findings are in agreement with the selenoprotein hierarchy, where GPX2 is located at the top position [Wingler, et al., 1999]. These findings also provide evidence that these transcripts are more sensitive to AG than to supplemental Se.

Whether and to what extent the AG affect the functionality of selenoenzymes remained elusive. Although Western blot analysis evidently showed that G418 promotes the biosynthesis of GPX and DIO1 (Figure 29, Figure 31) in Se-depleted HepG2 cells, stimulation with G418 or gentamicin resulted in a non-detectable alterations to enzyme activity, i.e. remained below the detection limit of this method. When Se-supplemented hepatocytes were stimulated with G418 or gentamicin, the GPX and DIO1 enzyme activity was comparable to the Se-supplementation control. As an aside, the importance of Se for the catalytic function of selenoenzymes was proven in Sec-substitution studies, wherein the substitution of cysteine for Sec resulted in a reduced catalytic efficiency of DIO1 [Berry, et al., 1993]. Collectively these findings demonstrate AG ability to induce the biosynthesis of selenoenzymes, although the selenoenzymes are not functional under Se-depleted conditions. We could conclude that a fraction of selenoenzymes synthesised are Se-free as found in Western blot analysis (Figure 29 A, Figure 31 B), furthermore the additional formed selenoenzyme fraction is not functional as shown in investigating the enzyme activity (Figure 29 B-D, Figure 31 A). We also may

conclude that there would be an AG effect, which as is known, is neutral with regard to the overall enzyme activity.

As G418 strongly induced the transcript level of nearly all analysed selenoproteins (Figure 27, Figure 30 and Figure 31), the question remained whether the increase in protein expression level results from increasing transcript levels. To answer this, an artificial cell model was taken advantage of. In this cell model, a FLAG-tagged cDNA of the human *DIO1* was cloned into a protein expression vector and stably overexpressed in HEK293T cells [Stoedter, et al., 2015]. The exclusion of the promoter regions and mRNA regulatory mechanisms allows isolated investigations on post-transcriptional regulation and protein stability. In this respect, chemotherapeutic selenocompounds, namely methyl- imidoselenocarbamates, were identified as strong post-translational inducers of the iodothyronine deiodinases [Stoedter, et al., 2015]. Western blot analysis and DIO1 enzyme activity assays revealed that G418 and gentamicin promote the DIO1 protein expression in a concentration-dependent manner in Se-deficient HEK293T cells, but the induced levels were still below the Se-control. Despite this strong induction in the protein expression level, the DIO1 enzyme activity in Se-deficient HEK293T cells was below detection limit of this method, and therefore in line with previous findings (Figure 32). Consequently, in Se-supplemented cells neither G418 nor gentamicin altered the protein level and DIO1 enzyme activity level. These results clearly show that AG impact the biosynthesis of selenoproteins during protein translation, but not the transcriptional level, supporting the conclusion that AG do not appreciably impair the Se-dependent selenoenzyme activity.

Although these finding demonstrate that the AG impact selenoproteins at the post-transcriptional level by inducing an UGA readthrough, it does not explain the observed AG-driven increase in selenoprotein transcription levels. As the AG-derived induction of selenoprotein expression can occur independent from the transcript level (Figure 32) and AG interfere irreversibly with the small ribosomal subunit, it is likely that this interference stabilises the mRNA and thereby protects it from NMD. Accordingly, rescue studies of the non-sense mutated p53 tumour suppressor gene have demonstrated that AG treatment stabilises the mutant mRNA, which would otherwise have been degraded by NMD [Floquet, et al., 2011]. NMD efficiency has furthermore shown to govern the response to gentamicin [Linde, et al., 2007]. The *GPX1* transcript is a preferred target of NMD in Se-deficiency, resulting in increased mRNA degradation, but Se-supplementation triggers the switch from NMD to a several-fold increase of *GPX1* mRNA [Weiss Sachdev and Sunde, 2001]. Consequently, selenoproteins such as *GPX1* are that exhibit a high NMD under Se-deficiency become protected from NMD and results in a greater increase in mRNA expression level when AG are applied. In

comparison, the *GPX4* transcript that is not a preferred target of NMD is less affected by this protective mechanism. To this point, we cannot explain what minor effect on the biosynthesis of selenoproteins these findings comprise. Further studies on mRNA and protein stability e.g. by actinomycin or cycloheximide, as well as sequence analysis may reveal selenoprotein-specific differences.

4.3. IL-6 and AG act synergistic on SEPP biosynthesis

In *in vitro* studies*,* we have thus far only investigated the isolated effects of IL-6, G418 and gentamicin on the expression of hepatic selenoproteins. In these *in vitro* studies, IL-6 decreased the SEPP expression in a concentration-dependent manner and are thus in accordance with the findings in a cohort of neonates with connatal infections. Nevertheless, while G418 and gentamicin induced the synthesis of SEPP in the *in vitro* experiments, no such correlation was found between gentamicin and SEPP in the neonatal children [Wiehe, et al., 2016]. We therefore wanted to investigate whether this differences might be caused by an interplay of IL-6 and AG thereby affecting SEPP expression.

The application of either G418 or gentamicin led to diminished IL-6-mediated decreases in the SEPP expression. It is noteworthy that G418 increased the SEPP biosynthesis to a greater extent than gentamicin, as described earlier (Figure 26). However, as the application of G418 promoted the biosynthesis of Se-deficient SEPP (Figure 28), the question emerged whether the diminishing effect, described above, might be a consequence from G418-induced biosynthesis of Se-deficient SEPP. In subsequent studies investigating the Se-load per SEPP molecule, we were able to confirm that the additional application of G418 reduces the amount of Se per SEPP molecule and irrespectively of IL-6 (Figure 34 C). As IL-6 down-regulates the SEPP expression, but does not alter the Se-load per SEPP molecule, it seems likely that the observed increase in the Se-deficient SEPP synthesis results from the G418-induced synthesis of Se-deficient SEPP. This would result in two possible SEPP isoforms, a Se-loaded SEPP isoform and Se-deficient SEPP isoform. The co-existence of both SEPP-isoforms seems likely as we could demonstrate an increase in the overall SEPP concentration (Figure 33), but a reduced molar ratio of Se per SEPP (Figure 34). This hypothesis is supported by similar findings of *Wiehe et al.* (2016), where the SEPP plasma concentration increased significantly in the infected group after antibiotic treatment, while the total plasma Se-supplementation remained unaffected (Figure 9 A+B) [Wiehe, et al., 2016].

5. Conclusion and results

This work describes studies on the molecular interplay between pro-inflammatory cytokines in particularly IL-6, aminoglycoside antibiotics, the Se-status and their synergistic effects on the expression of selenoprotein in liver cells allowing a better understanding and interpretation of severely decreased plasma concentrations of Se and SEPP during critical illness [Angstwurm, et al., 2007; Forceville, et al., 1998; Hollenbach, et al., 2008].

The Se-supply of peripheral organs, e.g. kidney or brain with Se, relies almost exclusively on Se-supply by liver derived SEPP. SEPP-mediated Se-transport is indispensable in maintenance of essential selenoproteins counteracting an increased production of ROS during the immune response. Since SEPP plays an important key role in the Se-distribution for the entire organism, it is of high importance to identify factors affecting the gene transcription or translation. In this regard, our study of newborns with connatal infections identified IL-6 and gentamicin as clinically relevant modulators of SEPP expression [Wiehe, et al., 2016]. Subsequent *in vitro* studies showed that both IL-6 and AG negatively affect the expression of functional SEPP, albeit by different mechanisms and in a different direction. While IL-6 directly induces a redistribution of hepatic selenoprotein expression in favor of antioxidative selenoproteins, e.g. GPX2, IL-6 greatly down-regulates the hepatic production of SEPP, resulting in a reduced circulating SEPP concentration (Figure 36 A) [Martitz, et al., 2015].

This work further illustrates the complexity of how AG interfere with the selenoprotein biosynthesis. The AG-induced misinterpretation of the UGA codon turned out to depends on the specific AG, the AG concentration, the selenoprotein-specific SECIS-element and the Se-status [Martitz, et al., 2016]. AG further promote the biosynthesis of Se-free selenoproteins and non-functional selenoenzymes. SEPP appeared to be the most sensitive selenoprotein to AG-treatment resulting in the large production of Se-free SEPP (Figure 36 B). However, the experiments studying an adjuvant Se-supplementation highlighted that a sufficiently high Se-status is able to diminish the negative effects of AG on the activity of selenoenzymes and regular biosynthesis of SEPP.

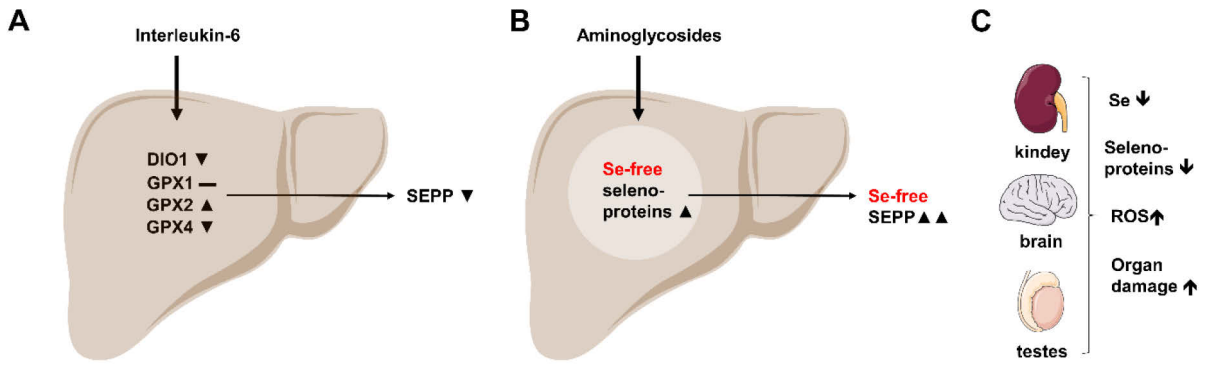


Figure 36: Impact of IL-6 and AG on selenoproteins in liver and consequences for Se-metabolism.

(A) IL-6 promotes the redistribution in hepatic selenoprotein expression in favour of antioxidative selenoproteins, while other selenoproteins are down-regulated. Decreased biosynthesis of SEPP by hepatocytes, results in lower plasma concentrations of SEPP. (B) AG promote the biosynthesis of Se-free selenoproteins, especially Se-free SEPP. (C) The combination of IL-6-mediated reduced SEPP expression and AG-induced synthesis of Se-free SEPP results in Se-deficiency and subsequently decreased selenoprotein expression in SEPP target organs including kidney, brain or testes. In inflammatory processes, this will consequently lead to an impaired selenoprotein expression in target cells and potentially an increased ROS level followed by organ damage and organ dysfunction (Figure was produced using Servier Medical Art).

In summary, my data demonstrate that IL-6 and aminoglycosides inhibit the selenoprotein biosynthesis directly and through different molecular mechanisms. These findings are of particular relevance for critical Se-deficient patients, as the elevated IL-6 concentrations in combination with an AG treatment may severely interfere with regular SEPP expression both by reducing its biosynthesis and by promoting the production of Se-free SEPP. The consequence of these effects is an insufficient Se-supply of the peripheral and endocrine organs, e.g. kidney, brain, testes, or bone. A reduced expression of dispensable and more essential selenoproteins may result (Figure 36 C). Sequel, the antioxidative defence systems may become insufficiently expressed and intracellular ROS levels may increase in these organs, potentially leading to organ damage and eventual organ failure as found in patients with severe sepsis [Duran-Bedolla, et al., 2014]. These results underline the importance of the Se-status for a sufficient and undisturbed selenoprotein expression, particularly in critical illness and under clinical antibiotic treatment.

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8. Publications

Wiehe L, Cremer M, Wisniewska M, Becker NP, Rijntjes E, **Martitz J**, Hybsier S, Renko K, Bührer C, Schomburg L (2016) Selenium status in neonates with connatal infection. Br J Nutr. 2016 Aug; 116(3):504-13.

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9. Eigenständigkeitserklärung

Hiermit erkläre ich, Janine Martitz, dass ich die vorliegende Arbeit mit dem Titel „Factors impacting the hepatic selenprotein expression in matters of critical illness“ selbstständig angefertigt und alle verwendeten Hilfsmittel und Literatur vollständig angegeben habe.

Zudem versichere ich, dass die hier vorliegende Arbeit noch in keiner Form bei einer anderen Prüfungsbehörde bzw. Universität eingereicht wurde und ich nicht im Besitz eines Doktorgrades bin.

Des Weiteren erkläre ich, dass mir die Promotionsordnung, sowie das von mir entsprechend angestrebte Promotionsverfahren bekannt sind.

Berlin, den Janine Martitz