universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„A combined subcellular proteomics and lipidomics approach to evaluate the influence of EGCG on LPS induced inflammation in U937 monocytes "

verfasst von / submitted by

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Angestrebter akademischer Grad / in partial fulfillment of the requirements for the degree of

Master of Science (MSc)

Wien, 2017 / Vienna, 2017

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 862
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Chemie
Betreut von / Supervisor:	Univ.-Prof. Dr. Christopher Gerner

Acknowledgements

First of all I would like to thank Univ.-Prof. Dr. Christopher Gerner for the great opportunity to complete my master thesis under his guidance and work in his group on such an interesting topic.

My special thanks go to Günter Walder, who helped me with all the work in the cell culture. Johanna Mader trained me in the field of proteomics, whereas Ammar Tahir taught me lipidomics and last but not least Rupert Mayer and Laura Niederstätter, who helped me with the measurements at the HPLC-MS/MS and showed me, how to interpret the data.

But it was the whole group, who welcomed me and always listened to my problems and gave me precious advice whenever possible.

Finally I want to thank my family and friends and especially my love Irene, who was supporting me the whole time in uncountable different ways and who made it possible, to choose and chase this dream in my own, personal way.

Abstract

Next to coronary heart diseases cancer is the second leading cause of death in Austria and worldwide. But cancer is not a uniform disease; it is the common term for a large group of related diseases. They all share the fact that due to various reasons healthy body cells start to transform into tumor cells with uncontrolled growth. Cancer is closely linked to oxidative stress and inflammation, especially chronic inflammation. The environment created during oxidative stress and inflammation is beneficial for cancer, thus cancer cells try to establish and maintain the state of increased oxidative stress and inflammation. Importantly, carcinogenesis is a multistage process, which can be reversed or completely terminated at each step. Especially phytochemicals seem to have a high potential to interfere with the process of carcinogenesis, as they are able to reduce oxidative stress and inflammation and generally alter cell signaling in a favorable, stabilizing way. Epigallocatechin-3-gallat (EGCG) is a phytochemical, which is mainly found in green tea. Its positive effect on inflammation and cancer is described in many empiric studies, but until now no general mechanism is described. The aim of this study was to use a combined proteomics and lipidomics approach to investigate the influence of EGCG on LPS induced inflammation in U937 monocytes. The cells were cultivated, differentiated with PMA towards macrophages and stimulated with LPS and EGCG according to a treatment plan. Afterwards the cells were harvested and split up in subcellular fractions, supernatant, cytoplasm and nuclear extract. The proteins were precipitated with ethanol, the liquid phase after protein precipitation of the supernatant was used for lipidomics analysis, all protein pellets for proteomics analysis. In the lipidomics part of this study, the cell signaling molecules eicosanoids were investigated. The lipids were cleaned and enriched using polymeric columns and measured with HPLC-MS/MS. After a tryptic digest, the proteins were measured with nano-flow HPLC-MS/MS. All data was analyzed using different bioinformatics tools. The results demonstrate, that the induction of inflammation in U937 monocytes with LPS was successful. The level of a large number of eicosanoids and pro-inflammatory cytokines and chemokines was significantly increased. The LPS induced effects were partially modulated with EGCG. EGCG alone showed no significant effect. Actually the present multi –omics study proved highly efficient and successful for the analysis of very low abundant signaling molecules. While it hardly identified direct effects of EGCG on the formation of the inflammatory mediator molecules under the given conditions, it will hopefully inspire future studies to better characterize molecular effects of EGCG on cancer cells.

Zusammenfassung

Nach koronaren Herzkranzgefäßerkrankungen ist Krebs die zweithäufigste Todesursache, sowohl in Österreich als auch weltweit. Aber Krebs ist keine einheitliche Krankheit, es ist der Sammelbegriff für eine große Gruppe ähnlicher Krankheiten. Sie alle haben die Gemeinsamkeit, dass sich aufgrund verschiedenster Ursachen gesunde Körperzellen in Tumorzellen mit unkontrollierbarem Wachstum umwandeln. Krebs ist eng verbunden mit oxidativem Stress und Entzündungen, vor allem chronischen Entzündungen. Auf der einen Seite profitiert die Krebszelle von der Umgebung, die durch oxidativen Stress und Entzündungen geschaffen wird, auf der anderen Seite versucht die Krebszelle den Zustand von erhöhtem oxidativen Stress und Entzündung zu etablieren und aufrecht zu erhalten. Die Karzinogenese ist ein mehrstufiger Prozess, der grundsätzlich auf jeder Stufe rückgängig gemacht oder auch ganz abgebrochen werden kann. Vor allem sekundäre Pflanzeninhaltsstoffe scheinen ein hohes Potenzial aufzuweisen, in die Karzinogenese einzugreifen, weil sie oxidativen Stress und Entzündungen reduzieren können und generell das Signalnetzwerk der Zelle wünschenswert stabilisieren und beeinflussen können. Epigallocatechin-3-gallat (EGCG) ist ein sekundärer Pflanzeninhaltsstoff, der hauptsächlich im Grüntee vorkommt. Sein positiver Effekt auf Entzündungen und Krebs wurde in zahlreichen Studien empirisch beschrieben, aber bis jetzt wurde kein genereller Wirkmechanismus publiziert. Das Ziel dieser Studie war es, einen kombinierten Ansatz aus Proteomics und Lipidomics zu verwenden, um den Einfluss von EGCG auf LPS indizierte Entzündung in U937 Monozyten zu untersuchen. Die U937 Zellen wurden kultiviert, mit PMA zu Makrophagen differenziert und mit LPS und EGCG anhand eines Behandlungsplans stimuliert. Danach wurden die Zellen geerntet und in die subzellulären Fraktionen Zellüberstand, Zytoplasma und Kernextrakt getrennt. Die Proteine wurden mit Ethanol gefällt. Die flüssige Phase nach der Ethanolfällung der Proteine des Zellüberstands wurde für die Lipidomics Analysen verwendet, alle Protein Pellets für die Proteomics Analysen. Im Lipidomics Teil der Studie wurden die Signalmoleküle Eicosanoide untersucht. Die Lipide wurden mittels Polymersäulen gereinigt, aufkonzentriert und mittels HPLC-MS/MS gemessen. Nach einem tryptischen Verdau wurden die Proteine mittels nano-Fluss HPLC-MS/MS gemessen. Alle Daten wurden mit verschiedenen bioinformatischen Programmen ausgewertet. Die Ergebnisse zeigen, dass die Induzierung von Entzündung in U937 Monozyten durch LPS sehr gut funktioniert hat. Die Menge von vielen Eicosanioden und entzündungsfördernden Zytokinen und Chemokinen wurde signifikant erhöht. EGCG modulierte diesen Effekt teilweise, hatte aber alleine keinen signifikanten Effekt. Diese Studie verwendete somit erfolgreich eine Kombination aus mehren –omics Bereichen und produzierte einen komplementären und konsistenten Datensatz, was eine Inspiration für weitere molekulare Studien sein kann.

Abbreviations

AA	Arachidonic acid
ABC	Ammonium bicarbonate
ACN	Acetonitrile
AUC	Area under the curve
BSA	Bovine serum albumin
COX	Cyclooxygenase
CSF	Colony stimulating factor
CYP 450	Cytochrome P450 monooxygenase
CYT	Cytoplasm
DGLA	Dihomo-γ-linolenic acid
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Epicatechin
ECG	Epicatechin-3-gallat
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallat
EPA	Eicosapentaenoic acid
EX	Eoxin
FA	Formic acid
FBS	Fetal bovine serum
FDR	False discovery rate
GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HCD	Higher-energy collisional dissociation
HEPE	Hydroxy/hydroperoxyeicosapentaenoic acid
HESI	Heated electrospray ionization
HETE	Hydroxy/hydroperoxyeicosatetraenoic acid
HHTrE	Hydroxy/hydroperoxyeicosatrienoic acid
HPLC	High-performance liquid chromatography
IAA	Iodoacetamide
IAP	Isotone dissociation buffer

IFN	Interferon
IL	Interleukin
LFQ	Label free quantification
LOX	Lipoxygenase
LPS	Lipopolysaccaride
LT	Leukotriene
LX	Lipoxin
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NE	Nuclear extract
O^{2-}	Superoxide anion
$OH^{\bullet}$	Hydroxyl radical
PCA	Principal component analysis
PG	Prostaglandin
PGI	Prostacyclin
PIC	Protease inhibitor cocktail
PMA	Phorbol-myristate-acetate
PMSF	Phenylmethylsulfonyl fluoride
PS	Penicillium/Streptomycin
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Retention time
SDS	Sodium dodecyl sulfate
SN	Supernatant
SOD	Superoxide dismutase
SPE	Solid phase extraction
TCM	Traditional Chinese medicine
TNF	Tumor necrosis factor
TX	Tromboxane
VEGF	Vascular endothelian growth factor

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1. Introduction

Cancer is undoubtedly one of the major diseases that pester humanity these days. It is second to coronary heart diseases the main cause of death in Austria and worldwide. Although the mortality of cancer is decreasing in the last years, the cases of cancer are increasing. In general, cancer is the common term for a large group of related diseases, which share the fact that healthy body cells transform into tumor cells in a multistage process. The tumor cells grow uncontrollably because the body loses the cell cycle control and cellular regulation. Cancer is closely related to oxidative stress and inflammation. Oxidative stress, the imbalance between reactive oxygen species and the body's own detoxification mechanisms, can promote the process of carcinogenesis by causing additional DNA damage. Inflammation is a natural, originally temporary response of the body to harmful stimuli in order to prevent further harm and repair already occurred damage. A lot of temporary changes in cell signaling are performed, and among others also the level of oxidative stress rises. Oxidative stress, inflammation and especially chronic inflammation can create an environment, which is beneficial for cancer cells and helps them to advance in the process of carcinogenesis. Because of this beneficial influence, cancer cells try to establish and maintain this process of increased oxidative stress and inflammation. Especially phytochemicals, as found in plant food, seem to have a stabilizing effect on cellular pathways, help the body to keep control over cellular regulations and cell cycle control and therefore repel cancer. Numerous studies prove the effect of various plants or plant extracts on cancer empiric, but finding and describing the exact mechanisms seems to be much more difficult. As for epigallocatechin-3-gallat (EGCG), a phytochemical which is mainly found in green tea, many empiric studies with a positive effect on carcinogenesis, oxidative stress and inflammation can be found. Also different mechanisms are postulated, but until now no general mechanism is described. Presumably due to technical limitations until now only studies which investigate selected proteins can be found in literature. A high resolution in solution shotgun proteomics approach to investigate the whole proteome in combination with lipidomics is novelty. Especially the combination of proteomics combined with lipidomics is beneficial, as the state of signaling pathways in a cell is not only determined by proteome alone, but also other signaling molecules like eicosanoids play a crucial role. The combination of both -omics methods is intended to give a better understanding of the whole situation. [Alberts *et al.*, 2002; Reuter *et al*., 2010; Rahmani *et al.,* 2015; Statistik Austria, 2017]

The aim of this study was to investigate the influence of EGCG on lipopolysaccharide (LPS) caused inflammation in U937 monocytes. Therefore U937 cancer cells were cultivated. After differentiation towards macrophages with phorbol-myristate-acetate (PMA) identical fractions were incubated with LPS and/or EGCG according to a treatment plan. (Con, +EGCG 20h, +EGCG 44h, +LPS 20h, +EGCG 44h +LPS 20h; see Table 1) The cells were harvested and after subcellular fractionation (SN, CYT and NE) the proteins were precipitated with ethanol. The liquids of the cell supernatant samples (SN) were used for lipidomics analysis. The cell signaling molecules eicosanoids were investigated. After cleaning and enriching the samples with polymeric columns (Strata X), the samples were measured contemporary with HPLC-MS/MS. The protein pellets of all three subcellular fractions were used for proteomics analysis. The whole proteome was investigated. The protein pellets were dissolved in sample buffer and transferred to Eppendorf tubes. After determining the protein concentration via Bradford assay, proteins were digested tryptic and measured with HPLC-MS/MS. All resulting data was analyzed with different bioinformatics programs (Xcalibur, Proteome Discoverer and Perseus).

2. Theoretical background

2.1. Oxidative stress, inflammation and cancer

Oxidative stress is an imbalance between free radicals and oxidants in a system and their elimination by protective mechanisms. In detail, the amount of reactive oxygen species (ROS) like superoxide anion(O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and organic peroxides and under certain circumstances also the amount of reactive nitrogen species (RNS) like nitric oxide (NO) is elevated. The body's own detoxification mechanisms, e.g. superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, catalase and non-enzymatic antioxidants like glutathione (GSH), vitamin C and vitamin D, are unable to decrease the amount of ROS to a normal level, so that they can react with biological molecules like proteins, lipids and DNA (deoxyribonucleic acid) and alter or inhibit their function. A certain amount of ROS is produced naturally in cells by the mitochondrial respiratory chain and plays an important role in cell signaling, but the body's own protective mechanisms normally can cope quite well with them. Oxidative stress occurs, when additional ROS are produced due to environmental stress like radiation, heat exposure and most important xenobiotics (e.g. tobacco smoke, pollutants in food and drugs). [Reuter *et al*., 2010]

In general, inflammation is a natural, complex biological response of the body to harmful stimuli. These stimuli can be physical (burns, frostbite, physical injury, radiation), biological (infections, stress), chemical (toxins, irritants)and even psychological. In order to prevent further damaging and repair already occurred damage, the immune system is activated, which results in the five cardinal signs of inflammation (pain, heat, redness, swelling and loss of function). In general, the damage is recognized by local immune cells and they release pro inflammatory vasodilators and chemotactic factors like histamine. This leads to increased capillary, permeability and blood flow to the area, which brings more leukocytes and mast cells to the damaged area. The immune system fights the damage, which uses more oxygen and leads therefore to an increase in the production of ROS. The body starts to repair the damage and anti-inflammatory markers are released, which calm down inflammation and the tissue starts to return to normal. This is an overall scheme for an acute inflammation, but due to different reasons, like e.g. continuing harmful stimuli, an inflammation can become chronic. This means that the temporary state of inflammation persists, which causes several problems for the body. Maintaining the state of inflammation for a long time is energy-consuming and wearing for the whole system, because the body starts to mobilize stored reserves and even shift energy from other bodily systems to the immune system. Furthermore often food intake and appetite are decreased during the state of

inflammation, as nearly every person has witnessed on himself. This worsens the energy shortage during chronic inflammation. Another problem is the increased production and accumulation of ROS. A temporary increase of ROS is tolerable or even beneficial for cause of fighting the damage, but as the state of inflammation continues and the ROS levels increase, they become more and more troublesome and even malignant. As already described above, when the body's own detoxification mechanisms fail to control the oxidative stress caused by the high levels of ROS, they start attacking and destroying the body's own biological molecules. Especially the reaction of ROS with DNA can completely invert cells functioning and this is one point, where oxidative stress and inflammation can support the process of carcinogenesis. [Reuter *et al*., 2010; Straub *et* Schradin, 2015]

Cancer is after coronary heart diseases the second leading cause of death. It was responsible for 8.8 million deaths in 2015 worldwide, which means 1 of 6 deaths worldwide is due to cancer. In Austria approximately 39000 new cases of cancer were documented in 2014 and approximately 20 000 people died because of cancer. This means, in 2014 cancer is responsible for 1 of 4 deaths in Austria. Statistic claims, 31 out of 100 males and 24 out of 100 females in Austria will suffer from cancer before they are 75 years old. In Austria in 2014, approximately 330 000 persons lived with the diagnosis cancer. More than half of all cases of cancer were colon-, lung-, breast- or prostate cancer. [Cancer Fact sheet, 2017; Statistik Austria, 2017]

Cancer is the common collective term for a large group of related diseases, where abnormal body cells grow unregulated and spread, invade and destroy healthy cells maliciously. On molecular biological sight it’s always a loss of cellular regulation and cell cycle control. There are six hallmarks of cancer, which are shown by every tumor cell. First, cancer cells grow and divide even in the absence of proper growth signals from other cells; they can produce stimulatory signals for themselves or even alter their extracellular matrix to produce these signals. Next, cancer cells grow and divide even when given contrary signals from other dells, they are insensitive to anti-growth signals. Related to this ability is the next hallmark, cancer cells are able to evade apoptosis. Body cells always check and control each other and if one cell is found to be abnormal (like cancer cells); it is given signals from the surrounding cells, which should lead to a programmed cell death (apoptosis). Cancer cells can show a variety of ways to ignore or dispose these signals, but the most common and frequent are mutations in the p53 tumor suppressor gene. The next hallmark is the limitless potential of replication of cancer cells. Normal cells only can divide a limited number of times due to the shortage of the telomeres. Tumor cells need to solve this problem to divide uncontrollable, e.g. by turning on telomerase. Sustained angiogenesis is the

fifth hallmark of cancer cells. Due to their ongoing growth and division, cancer cells need lots of oxygen and nutrients and so they found a way to stimulate the growth of blood vessels. The sixth hallmark of cancer is their capability of tissue invasion and metastasis. Cancer cells grow and divide continuously, so the tumor grows bigger and bigger and sooner or later it starts to spawn tumor cells, which move through the blood circulation system and try to find new places, where they invade and then start to grow, divide and form a new tumor (metastasis). [Alberts *et al.*,2002; Hanahan *et* Weinberg, 2000; Reuter *et al*., 2010]

Cancer and carcinogenesis is a multistage process and is subdivided in three stages: initiation, promotion and progression (Figure 1). Oxidative stress and chronic inflammation can influence and advance all of these steps in various ways. At the step of initiation, ROS or other carcinogens can cause a persistent DNA damage, which must not be repaired by the body's own DNA repair systems or lead to apoptosis. Typical targets for this first mutation are genes, which control cell cycle and proliferation, like tumor suppressor genes. After this first mutation, of course additional mutations can follow. During promotion in carcinogenesis, ROS can alter and disturb various cell signaling pathways. The alteration always has to promote the growth and proliferation of the initiated tumor cells. For example during inflammation growth factors are produced to stimulate cells which repair damage, but of course initiated tumor cells can also use these growth factors to boost their own growth and proliferation. So during chronic inflammation, the growth of initiated tumor cells is promoted and they naturally pass on all their accumulated DNA mutations to their daughter cells. During the last step of carcinogenesis, progression, further DNA alterations are caused by ROS or other carcinogens. With all their accumulated mutations the tumor cells lose their normal functions, they are immortal and proliferate continuously. They become malicious, which means they invade surrounding tissues and start metastasizing. Oxidative stress and inflammation create an environment, which supports cancer cells in their advance in carcinogenesis, but cancer cells themselves try to create and maintain this state of oxidative stress and inflammation. [Alberts *et al.*, 2002; Klaunig *et al.*, 1998; Reuter *et al*., 2010]

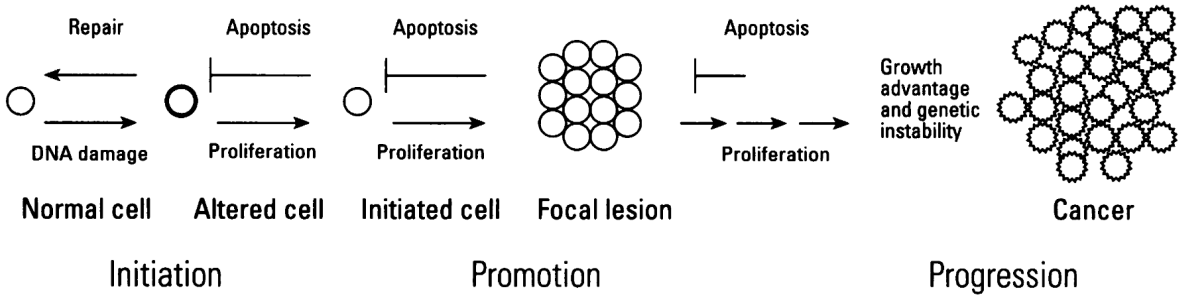

Figure 1: Multistep process of carcinogenesis [Klaunig *et al.*, 1998]

As described above, cancer is not a single defined state; it is a variable multistep process. This process can be influenced not only by oxidative stress and inflammation, our whole diet and lifestyle can have a major impact on cancer. Studies estimate, that only 5-10 % of all cases of cancer originate in a genetic predetermination, 90-95 % of all cases of cancer have their roots in environment and lifestyle. Among cigarette smoking, alcohol consumption, sun exposure, environmental pollutants, infections, stress, obesity and physical activity, especially diet seems to be a major environmental factor, which influences cancer. Approximately one-third of all cancer related death is due to diet, another third due to smoking and the last third distributes to all the other factors. Diet is such an important factor, because it can influence most of the other factors. This is obvious for obesity and the intake of environmental pollutants and infectious agents, but diet can also alter, lessen or even worse the negative effects of cigarette smoking, alcohol consumption and sun exposure. When it comes to diet, a high percentage of plant food and in general lowering energy consumption paired with increased physical ability seems to be beneficial. The intake of energy dense food (fat, sugar and also alcohol), red meat and processed meat, salt and other dietary supplements should be limited. This does not conclude, that these products should be completely avoided, just lowering their percentage, as it is too high in western style diet, seems to be ideal. [Anand *et al.*, 2008; Wicki *et al.*, 2011; World Cancer Research Fund, 2007]

2.2. Green tea

Green tea is an aqueous extract of briefly steamed or roasted and then dried leaves from *Camellia sinensis.* Although it is most popular in Asian countries like China, India and Japan, green tea is consumed worldwide and after water, green tea is the most consumed beverage in the world. People in China drank green tea already before the Nativity. In these early times green tea was mainly known as a medicine. In Traditional Chinese medicine (TCM) many positive effects of green tea on body and soul like strengthening of circulation and immune system are described. But of course people also recognized the good taste of green tea and it became a popular luxury food. [Haller-Zingerling, 2010]

2.2.1. Constituents

A cup of green tea consists of approximately 99.65 % water and 0.35 % extracted solids. Naturally these values are influenced by brewing time, water temperature and amount and of course composition of the used tea leaves. The amount of extracted solids is thereby stronger influenced than the composition of extracted solids. The extracted solids consist of approximately 30 % catechins, 7 % sugars, 6 % proteins, 3 % lipids and 3 % caffeine. The most important catechins in green tea are epicatechin (EC), epicatechin-3-gallat (ECG), epigallocatechin (EGC) und epigallocatechin-3-gallat (EGCG). The structure of these catechins is displayed in Figure 1. EGCG seems to be the most effective catechin in green tea. Besides catechins, there are a large number of other polyphenols and other phytochemicals, which can be detected in various amounts in green tea. [Harbowy *et al*., 1997]

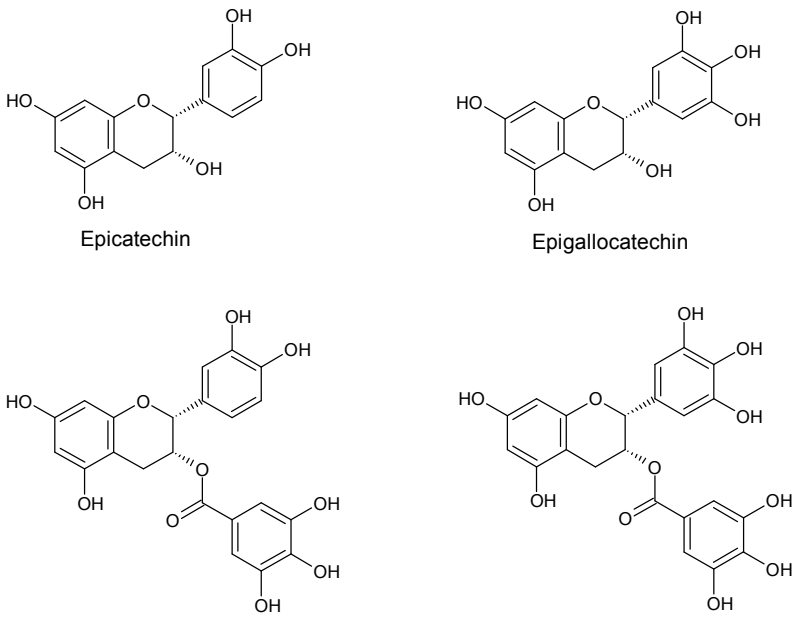

Figure 2: Main catechins in green tea

2.2.2. *Effects of green tea*

Phytochemicals in general are supposed to have an effect on the functioning of cells and therefore have the ability to influence human health. Up to now, only partial but no general mode of action is described. Various epidemiological studies seem to prove, that the ongoing intake of an array of phytochemicals with synergistic and antagonistic effects seems to have a positive and stabilizing influence on cells and cell systems. Green tea contains different phytochemicals in various amounts and so this general positive effect on human health was described for green tea in various papers. Green tea has chemoprotective properties and can help to prevent cancer or delay its progression. Furthermore green tea and its constituents play a role in the prevention of cardiovascular diseases, because green tea has antioxidative and radical scavenging, antihypertensive and antidiabetic properties and can help to reduce body weight. Other beneficial health effects of green tea are its antibacterial properties, protection against ultraviolet rays and its inhibition of caries.[De Bacquer *et al.,*2006; Hakim *et al.*, 2004; Na *et* Surh., 2007; Srinivasan *et al.*, 2004; Yang *et* Hong, 2013]

In various studies the modulation of cell signaling pathways by green tea via the following modes of action is described. It promotes the transcription of tumor suppressor genes like p53. Green tea and especially EGCG can induce apoptosis and delay the angiogenesis of tumors via inhibition of the vascular endothelian growth factor (VEGF). Furthermore there is an effect of green tea on enzymes of the drug detoxification metabolism. The transcription of phase I enzymes, which further process drugs in our body and can lead to toxification of drugs, are inhibited and the transcription of phase II enzymes, which are conjugated to drugs to excrete them easier, are promoted. Studies claim, that the activities of cyclooxygenase 2 (COX-2) and lipoxygenase (LOX) are reduced and green tea can influence the enzymes of the Akt-pathway via promotion of the activity of e.g. caspase-9. Moreover green tea can inhibit the activity of growth factors and their receptors like HER2, HER3 and EGFR and thus slow down tumor growth. Also the activity of mitogen-activated protein kinases (MAPK) is reduced by green tea and its main effective component EGCG. In literature, various other pathways, which are influenced by green tea and especially EGCG, are described. To review all these effects in a short manner, green tea can influence signal transduction in a cell. It can slow down cell cycle and tumor growth, induce apoptosis and seems to have a positive effect on health when consumed regularly, as proved in many studies. [Khan *et al.,* 2006; Mukhtar *et* Ahmad, 2000; Rahmani *et al.,* 2015]

2.3. Lipids and lipidomics

2.3.1. Lipids

Lipids are a group of naturally accruing molecules with a big variety of structures, properties and biological functions. A loose definition is, that lipids are a class of biological molecules, which are mostly insoluble in water, but in many cases soluble in organic solvents and have areas consisting of long carbon chains. A newer and more scientific definition is: "Lipids may be broadly defined as hydrophobic or amphipathic small molecules that originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units." (Citation from: The LIPID MAPS Lipidomics Gateway - Tutorials and lectures on lipids, www.lipidmaps.org/resources/tutorials/lipid_tutorial.html#L) Lipids are divided into eight major groups in this definition: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids (Figure 3). Each major group is partitioned into subclasses, but all of the subclasses share a common basic structure. [Christie 2017; Fahy *et al.,* 2005]

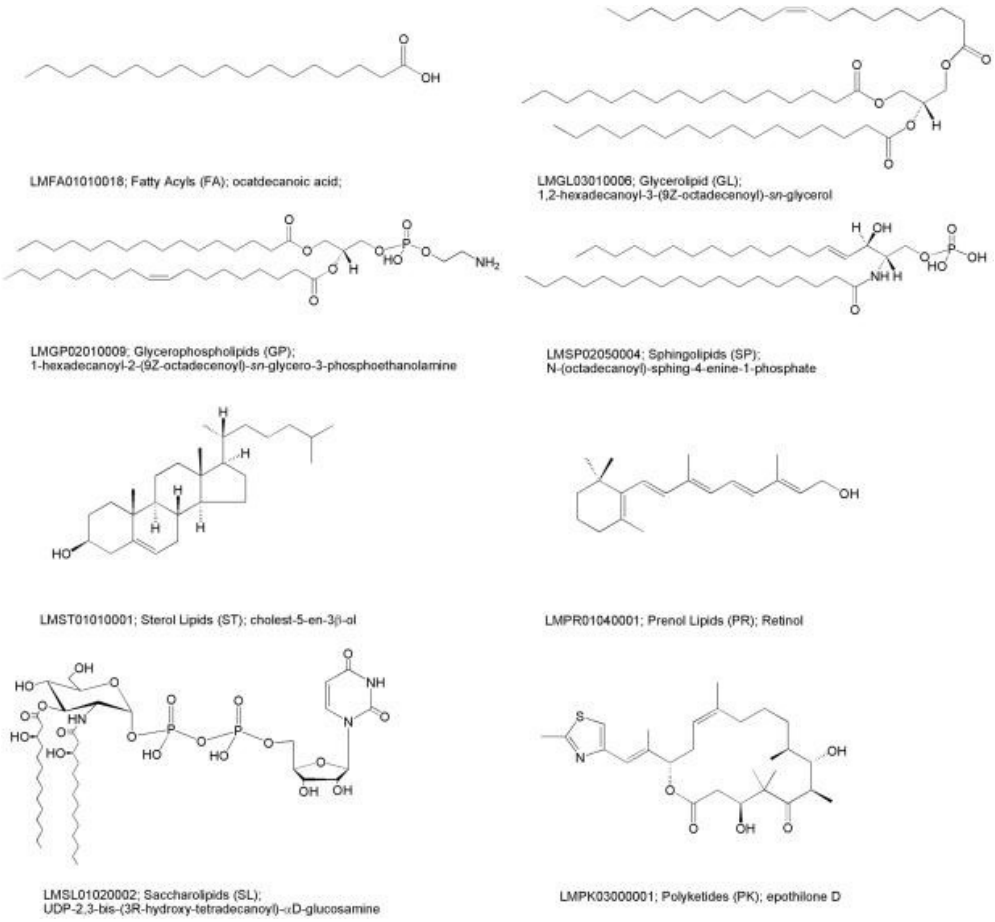

Figure 3: Structures of the eight major lipid groups with an example of each group [Sud *et al.,* 2006]

Lipids play an important role in nearly all biological processes. They form lipid bilayers, which act as multifunctional barriers between cell compartments and to the cell exterior. But furthermore lipids are used for energy storage. The complete oxidation of fatty acids provides about 9 kcal/g energy, compared to about 4 kcal/g energy from carbohydrates and proteins. A newer finding is that lipids play an important role in cell signaling and therefore are involved in cell growth, immune system, inflammation and many diseases. [Christie, 2017]

2.3.1.1. Eicosanoids

Eicosanoids are signaling molecules containing 18 to 22 (mostly 20) carbon atoms. They are synthesized by enzymatic or non-enzymatic oxidation of polyunsaturated fatty acids (PUFAs). Eicosanoids show a great variety in structure, because there are different PUFAs like arachidonic acid (AA, 20 carbon atoms), eicosapentaenoic acid (EPA, 20 carbon atoms) and dihomo-γ-linolenic acid (DGLA, 20 carbon atoms) that substrate for oxidation by different enzymes or even non enzymatic oxidation by reactive oxygen species (ROX). The most common enzymes are cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 monooxygenases (CYP 450). Normally these enzymes oxygenate one double bond of the substrate, so there are different possible positions, but the resulting eicosanoids are similar and belong to the same subclass. Furthermore the resulting eicosanoids can be further processed by downstream enzymes, which increases the variety. Eicosanoids are divided in several subclasses by structural similarity and origin. The most important subclasses are leukotrienes (LT), eoxins (EX), hydroxy/ hydroperoxyeicosatrienoic acids (HHTre, HETrE), hydroxy/hydroperoxyeicosatetraenoic acids (HETE), hydroxy/hydroperoxyeicosapentaenoic acids (HEPE) and prostanoids, which are further divided into prostaglandins (PG), prostacyclins (PGI), lipoxins (LX) and thromboxanes (TX). An example of some eicosanoid subclasses is displayed in Figure 4.[Christie, 2017; Dennis *et* Norris, 2015]

Eicosanoids function as signaling molecules and they can modulate a wide array of biological processes, like cell proliferation and apoptosis and the response to exterior and interior stimuli. As a response to other stimuli, eicosanoids can alter immune responses and cause or suppress inflammation, and more concrete they are responsible for e. g. fever, blood coagulation and blood pressure. Eicosanoid expression levels are altered in many diseases like atherosclerosis, bronchial asthma, multiple sclerosis and cancer. Therefore, some of the most successful drugs like Aspirin (acetylsalicylic acid) and Ibuprofen target eicosanoids and eicosanoid formation. [Astarita *et al*, 2015; Christie, 2017; Dennis *et* Norris, 2015]

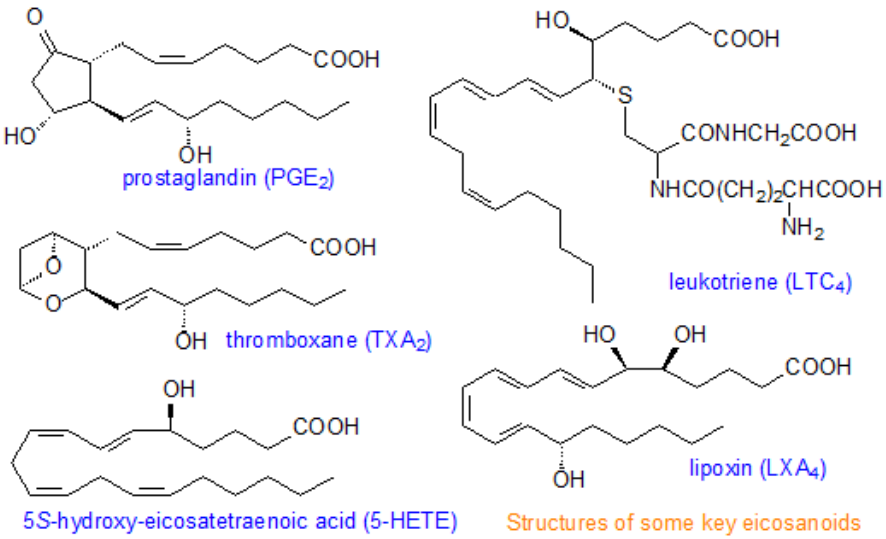

Figure 4: Examples of some eicosanoid subclasses [Christie, 2017]

2.3.2. *Lipidomics*

Lipidomics is the investigation of all or selected lipids in a biological sample and their metabolism, pathways and biological aspects, like e. g. diseases. Lipidomics is the one of the youngest of the -omics research fields, but its importance is growing as people recognize that not only genes and proteins determine the state of biological systems. Often small messenger molecules in low concentrations like eicosanoids can change a genomic predisposition and alter a proteomic state and so they can determine the course of diseases. Lipidomics are not as sophisticated and fully automated as established genomics and proteomics and bioinformatics and analysis tools are not as common as in proteomics or genomics. Some bioinformatics programs can be adapted for lipidomics, but a lot of analysis work has to be done manually. This is one of the reasons which make lipidomics difficult and time consuming. Furthermore the nature of lipids and in detail eicosanoids raises some special challenges, compared to other -omics fields. [Christie, 2017; Han, 2016]

The first is the hydrophobicity of lipids. Normal tubes and tips are designed to work with aqueous solutions and are often made of polypropylene or other plastics. Lipids can stick to the walls of tubes and tips and get lost for analysis. Often organic solvents are necessary to extract and dissolve lipids. But these organic solvents also have drawbacks. Normally they are more expensive and harmful to health and environment than aqueous solvents and they therefore often need special disposal. But the biggest drawback is that concentrated organic solvents can dissolve plastic tubes and tips and therefore contaminate the samples. It would be ideal to use glass instead of plastic, but glass has other drawbacks, e. g. limited centrifugation. [Han, 2016]

The next challenge in lipidomics is the isobaricity of many lipids and specially eicosanoids. Most eicosanoids contain 20 carbon atoms and they are derived from a few different PUFAs. Often one of several double bonds in a molecule is oxygenated, so all of the resulting different eicosanoids have the same molecular weight. With high quality HPLC it is possible to at least partially separate these molecules and it is even possible to separate different enantiomers. In mass spectrometry, discrimination is only possible via MS^2, because mass analyzer separate molecules due to their molecular weight. If these similar molecules are fragmented, they produce different fragmentation patterns and so different isobaric molecules can be distinguished. Normally different fragments are generated, but sometimes only the intensity of some fragments varies, which acquires high resolution measurements and high quality reference spectra measured under similar conditions to assign the spectra to the right substance. [Han, 2016]

Another problem is the instability of many lipids. They can easily decay or become further oxygenated by ROS or O_2 if they are not handled with caution. It is advisable to reduce dwell time to a minimum, work whenever possible on ice and store samples if it cannot be avoided on -20 °C. Because of this known problem with stability of lipids, it is necessary to treat al samples the same way, so if some lipids are lost during analysis, the percentage of lost lipids is at least nearly the same in all samples. [Han, 2016]

Interesting lipids and in detail eicosanoids are often found in low concentrations, which can complicate analysis. On the one hand enrichment via e. g. solid phase extraction (SPE) or evaporation of solvents is necessary to reach concentrations, which can be measured, on the other hand these enrichment steps can lead to a partial loss of the more instable lipids (decay or oxygenation) or lipids with certain hydrophobicity (stick to filter, tubes). [Han, 2016]

Because of all these problems, absolute quantification is difficult and cost intensive. An internal and external standard for each substance of interest would be necessary. Relative quantification, the change of the amount of a substance in a sample compared to a control of reference, is easier, cheaper and it at least partially compensates some possible errors. An internal standard is added to each sample at the beginning of the analysis and all measured values of each sample are divided through the measured value of the internal standard. If there is a loss of lipids in one sample, this loss can at least partially be compensated because we assume that there is a similar loss in the internal standard. Of course that is

only an approximation and therefore there is the relative quantification. If there is an unusual loss of a substance because of its physical and chemical properties, it can be assumed that the loss is similar in two different samples and by dividing the measured value of one sample trough the measured value of another sample, the individual variance of each substance is countered. The best example is the different ionization efficiency of different lipids. In mass spectrometry, the same amount of molecules can produce different amounts of ions during ionization depending on their chemical structure. Among others, this problem is solved by relative quantification. [Han, 2016]

2.4. Proteins and proteomics

2.4.1. Proteins

Proteins are macromolecules, which are formed by amino acids via peptide bonds. The amino acid sequence of all proteins is encoded in the genetic code of the DNA. This DNA code is transcribed into RNA and then translated into proteins. The newly processed RNA can be modified before translated into proteins via splicing and other processes, but it is also common to modify the newly synthesized proteins via post translational modifications. Proteins mainly consist of carbon, oxygen, hydrogen and nitrogen, but also other elements like sulfur in small amounts. Proteins with an amino acid count lower than about 50 are called peptides. Proteins are the main constituents of all cells; normally about 50 % of the dry weight of cells are proteins. Besides forming cells with their structure, proteins are the molecular machines of all cells. They transport molecules and ions, catalyze reactions, recognize and respond to exterior and interior stimuli and synthesize and catabolize biological molecules like DNA and ribonucleic acid (RNA), carbohydrates, lipids and also other proteins. [Alberts *et al.,* 2002]

2.4.1.1. Cytokines and chemokines

Cytokines are a group of small proteins (~5–20 kDa, ~ 100-200 amino acids), that play an important role in cell signaling. Normally they occur in low concentrations (picomolar), but as response to exterior or interior stimuli their concentration can be increased temporary up to about thousand fold. Cytokine signaling can influence the same cell (autokrine), neighbor-cells (parakrine) or even cells, which are reached via the circulatory system (endokrine). The effect of cytokines depends on their concentration and on the type of responsive cell. Furthermore the effect of cytokines is normally not determined via one cytokine alone, the ratio between different cytokines (pro and anti a specific effect) is important. Different cytokines can work together to trigger a response or work against each other and annihilate each other. Cytokines are secreted by a broad range of different cells, but due to their important role in the immune system, especially important cytokine producers are white blood cells like macrophages,

B-lymphocytes and T-lymphocytes. Beside their important role in the immune system, cytokines also influence maturation, growth and survival of cells. To be able to detect and response to cytokines, it is necessary for responsive cells to possess matching cell surface receptors. The number of these receptors can vary due to different circumstances and for example it can also be altered via cytokines, which is one way how cytokines influence themselves or other cytokines. Historically cytokines are divided into different subclasses like interferons (IFN), interleukines (IL), colony stimulating factors (CSF), tumor necrosis factors (TNF) and chemokines. A new approach classifies cytokines due to their structure and matching receptors. The subfamilies are IL-1-like cytokines (e. g. IL-1β), common γ-chain cytokines (e. g. IL-2), common β-chain cytokines (e. g. IL-3), IL-6-like cytokines (e. g. IL-6), IL-10-like cytokines (e. g. IL-10), interferons (e. g. IFN-β), tumor necrosis factors (e. g. TNF-α), transforming growth factor-β (TGF-β, e. g. TGF-β1) and chemokines. The latter have a special status among cytokines, because chemokines can induce directed chemotaxis in responsive cells. This process is especially important for immune response. Pro inflammatory chemokines attract leukocytes and direct monocytes, neutrophils and other effector cells from the blood to the place, where they are needed. Furthermore chemokines stimulate these cells to initiate an immune response or repair of the damage.[Arai *et al.*, 1997; Cameron *et* Kelvin, 2000-2013]

2.4.2. Proteomics

Proteomics is an important, well-established research method in modern molecular biology which focuses on analyzing the current proteome (total protein expression) or interesting parts of a selected system at a certain time point under defined circumstances. The aim of proteomics studies is to understand the structure, function and interaction of the proteome in the defined system and also compares systems with different circumstances to learn, how these exterior stimuli influence the proteome. For example, in this study the proteome of U937 cells before and after the application of drugs (LPS,EGCG) is compared to study the influence of these drugs. Recently proteomics analysis are often combined with other -omics analysis as lipidomics and metabolomics, as people recognize that especially these small molecules in low concentrations often have a large input on the state of the biological system. Especially modern MS-based proteomics is often a large scale, highly sophisticated and automated process, which would not be possible without the application of advanced bioinformatics. A wide array of bioinformatics programs and tools are available to support researchers and make the analysis of often large amounts of data less time consuming. There are two important approaches in proteomics. The first are immunoassays, which use antibodies to detect proteins. Naturally, this approach is only possible for selected, well known proteins because for each protein of interest, an

antibody with high specificity is necessary. The second approach is the use of mass spectrometry. To give a very short resume, the proteins are extracted and digested, and the resulting peptides are separated via chromatography. Normally the chromatography is on line coupled to the ion source of the mass spectrometer, where the sample is vaporized and the peptides are ionized. The ions are separated according to their mass to charge ratio via mass analyzers (e. g. quadrupole, ion trap, time of flight-analyzer, etc.). At this step tandem mass spectrometry (further fragmentation of selected ions) is possible. After that the ions hit a detector (e. g. electron multiplier) and the signals are transferred to electric current. The given mass spectra are compared to a database of in silico digested proteins and a list of proteins with their possibility to occur in the sample is calculated. By further analysis with bioinformatics tools and under special circumstances it is possible to absolute quantify the proteins, but also often only a relative quantification (comparison between two samples, e.g. before and after treatment) is sufficient. [Cañas *et al.*, 2006; Dzieciatkowska *et al.*, 2009]

3. Materials and methods

3.1. Cell culture

3.1.1. Handling and treatment

The human lymphoma model cell line U937 is cultivated in RPMI 1640 cell culture medium (RPMI 1640, Gibco Life Technologies Austria) with 10 % fetal bovine serum (FBS, Sigma-Aldrich) and penicillium/streptomycin (PS, ATCC Germany) in T25 cell culture flasks (BD Falcon™ Cell Culture Flasks25 cm^2, BD-Bioscience Europe) in an incubator (Heracell 150i CO_2Incubator, Thermo Scientific) at 37 °C and 5 % CO_2. Cells are counted automatically with MOXI Mini automated cell counter (ORFLO, USA) and 5 million cells are seeded in new T25 cell culture flasks. To initialize the differentiation of the U937 cells, PMA (Phorbol 12-myristate 13-acetate, Sigma Aldrich) at a final concentration at 100 ng/ml is added and the volume is filled up at 5 ml with cell culture medium. After 24 h the cell culture medium is replaced with a new medium without PMA. Therefore the cells are pipetted in a 15 ml tube (Falcon tubes, Corning Scientific) and they are centrifuged at 1100 rpm at room temperature for 5 min(Megafuge 16R Centrifuge, Thermo Scientific). After discarding the supernatant, the remaining pellet is resuspended in cell culture medium and it is transferred back to the cell culture flask and cell culture medium is added to a final volume of 10 ml. Then the cells are cultivated for another 72 h to fully differentiate. After that the five different fractions are treated accordingly to the treatment plan in Table 1. In all samples the old cell culture medium is replaced with fresh one like described above, but in the samples “EGCG 44 h” and “EGCG 44 h +LPS 20 h” EGCG (Sigma Aldrich) to a final concentration of 1 µM is added. All the samples are incubated for 24 h and afterwards the cell culture medium is replaced with fresh medium again. To the samples “EGCG 20 h” and “EGCG 44 h” EGCG at a final concentration of 1 µM is added. LPS (Sigma Aldrich) to a final concentration of 1 µg/ml is added to “LPS 20 h” and EGCG to a final concentration of 1 µM and LPS to a final concentration of 1 µg/ml are added to “EGCG 44 h +LPS 20 h”. After an incubation of 20 h the cell culture medium is removed and the cells are washed with 10 ml PBS. 6 ml serum-free RPMI cell culture medium are added and the cells are incubated for a final 4 h. [Sundström *et al.*, 1976]

Table 1: Treatment scheme of U937 cells

	24 h	72 h	24 h	20 h	4 h
Con	PMA	-	-	-	serum free
EGCG 20 h	PMA	-	-	EGCG	serum free
EGCG 44 h	PMA	-	EGCG	EGCG	serum free
LPS 20 h	PMA	-	-	LPS	serum free
EGCG 44 h + LPS 20 h	PMA	-	EGCG	EGCG + LPS	serum free

3.1.2. Cell fractionation

During the cell fractionation, the cells and the reagents are kept on ice at 4 °C whenever possible. After 4 h of serum free incubation, the cells are transferred to a 15 ml tube and they are centrifuged at 4 °C, 1800 rpm for 5 min. In the meantime the PIC (protease inhibitor cocktail, 880 µl H2O, 100 µl Pepstatin A (Sigma Aldrich), 10 µl Aprotein (Sigma Aldrich); 10 µl Leupeptin (Sigma Aldrich))and IAP (isotone dissociation buffer) + 1/100 PIC and +1/100 phenylmethylsulfonyl fluoride (PMFS, Sigma Aldrich) are prepared freshly. The liquid after the centrifugation (6 ml per sample) is diluted with 4 times cold ethanol, mixed and then stored over night at -20 °C to precipitate the proteins. These are the supernatant (SN) samples. 1 ml of the IAP is pipetted to the empty cell culture flask and the whole bottom of the cell culture flask is scrapped with a scrapper to resolve sticky cells. The whole solution is transferred to the pellet after the centrifugation. After dissolving the pellet, the liquid is resuspended 6 times against the wall of the tube to break open the cell membranes. After centrifugation (4 °C, 1800 rpm, 5 min), the liquid is diluted with 4 times cold ethanol and stored at -20 °C. These are the cytoplasm (CYT) samples. The remaining pellet is carefully dried with tissue paper, 100 µl TE NaCl (1 ml 1 M Tris/HCl + 0.2 ml 0.5 M EDTA + 10 ml 5 M NaCl + 88.8 ml H_2O) are added and the liquid is mixed by resuspending. After 10 min incubation, 900 µl TE MP40 (TE MP40 [1 ml 1 M Tris/HCl + 0.2 ml 0.5 M EDTA + 5 ml 10 % NP-40 + 93.8 ml H_2O] +1/100 PIC +1/100 PMSF, freshly prepared) are added and mixed by vortexing. The dissociation buffers react for another 15 min and then the samples are centrifuged at 4 °C and 3500 rpm for 5 min. The liquid after the centrifugation is diluted with 4 times cold ethanol and then mixed and stored at -20 °C overnight. These are the nuclear extract (NE) samples.

3.2. Lipidomics

3.2.1. Sample preparation

The proteins of the different cell fractions (SN, CYT, NE) in 15 ml tubes are precipitated with 4 times the volume of cold Ethanol at -20 °C overnight.5 µl of internal lipid standard mix (PGF2a-d4 standard (Cayman Europe), 15S-Hete-d8 standard (Cayman Europe), PGE2-d4 standard (Cayman Europe) are added to the samples and then they are centrifuged at 5000 rpm at 4 °C for 30 min with acceleration of 9 and deceleration of 4 (Megafuge 16R Centrifuge, Thermo Scientific). Immediately after the centrifugation is finished the liquid is poured off. The liquids of the cell supernatant samples (SN) are collected in 15 ml tubes and they are reduced by vacuum centrifugation(miVac Duo Concentrator, GeneVac) at 37 °C to about one-fifth of their original volume. If there are more 15 ml tubes of the same fraction, they are united and transferred to a 50 ml tube and four times the amount of LC-MS grade H_2O are added. The samples can be stored at 4 °C for a short time. The polymeric columns (Strata-X 33 µm polymeric reversed phase cartridges, Phenomenex Inc.) are placed on the vacuum-chamber and they are washed with 2 ml Methanol (MeOH) and then equilibrated with 2 ml LC-MS grade H_2O. The vacuum should be adjusted, so that there is a drop every 2-3 seconds and the columns should never dry completely. The samples are loaded on the columns and then the lipids are eluted from the columns with two times 250 µl of 49.5 % MeOH, 49.5 % Acetonitrile (ACN) and 1 % formic acid (FA) with a syringe in a HPLC-glass vial and they are reduced to nearly complete dryness by vacuum centrifugation at 37 °C. The remaining lipids are dissolved in 100 µl of 30 % ACN, 0.02 % FA, the vials are vortexed and the whole liquid is transferred to a V-shape HPLC-glass vial. Together with a blank (30 % ACN, 0.02 % FA) the samples are brought to the HPLC-MS/MS and are measured contemporary.

3.2.2. HPLC-MS/MS

All samples are measured by HPLC-MS/MS in technical duplicates. The HPLC is a Vanquish UHPLC by Thermo Fisher Scientific with a Kinetex® 2.1 mm x 15 cm, 2.6µm particle size, C18 reversed phase column by Phenomenex. The mobile phase A consists of 100 % LC-MS grade H_2O and 0.02 % FA, mobile phase B of 90 % ACN, 10 % MeOH and 0.02 % FA. A constant flow rate of 250 µl/min and a 45 min gradient (0-3 min: 35 % mobile phase B, 3-35 min: 35-98 % mobile phase B, 35-41,9 min: 98 % mobile phase B, 41,9-42 min: 98-35 % mobile phase B, 42-45 min: 35 % mobile phase B) are applied. 20 µl of each sample are injected and each sample is analyzed in technical duplicates. The HPLC is coupled to the heated electrospray ionization (HESI) source of a HF Q-Exactive Orbitrap mass spectrometer by Thermo Fisher Scientific. All scans are performed in negative ion mode. The m/z range for $MS^1$is 250-700 with a resolution of 30 000. The five most abundant ions undergo higher-energy collisional dissociation (HCD)-

fragmentation and are then measured in the orbitrap with a resolution of 15 000. To minimize the amount of unprofitable data, only ions whose mass matches with the mass of eicosanoids on an inclusion list, are fragmented. For this exact mass, a maximal deviation of +/- 5 ppm is allowed.

3.2.3. Data interpretation and bioinformatics

The resulting raw-files from the HPLC-MS/MS analysis are opened with Xcalibur (Xcalibur™, Thermo Fisher Scientific) and they are analyzed manually. The exact mass, RT (retention time) and AUC (area under the curve) of each known Eicosanoid on the inclusion list used before is excerpted to Excel sheets. It was planned to use measured MS^2 spectra and reference spectra from Lipidmaps (www.lipidmaps .org) to identify the peaks. This was not possible, because nearly no MS^2spectra were measured. The reason for this is that the maximal deviation for masses of the inclusion list was set to +/- 5 ppm. This value was too small; the real deviation was higher most of the time, so the software decided to not choose them for fragmentation and MS/MS analysis. This problem was solved by creating a list of previously identified eicosanoids and their RT in other experiments performed in this group. (not shown) Only experiments were chosen with the same or at least comparable HPLC conditions (same column, same mobile phase system, comparable gradient). With this list and the exact mass from the MS^1 scans RT-alignment was performed (same exact mass + same RT = same substance). This resulted in a list of totally 17 eicosanoids (3 standards) with their exact mass, RT and AUC for each treatment category and each technical and biological replicate. Eicosanoids with a RT smaller than 15 min were normalized to the standard 5S-HETE-d8, eicosanoids with a RT bigger than 15 min to PGF2α-d4. This was done by dividing the AUC of each Eicosanoid trough the AUC of the according standard with the same treatment. An average of the two technical replicates is calculated. Average and standard deviation of the three biological replicates, and when compared to other treatments, statistical significance (p-value) and fold change are calculated. The normalized average AUC and the according standard deviation are displayed in graphs created with GraphPad Prism (GraphPad Prism 6.07, GraphPad Software).[Fahy *et al.*, 2007; Sud *et al.*, 2006]

3.3. Proteomics

3.3.1. Sample preparation

The proteins of the different cell fractions (SN, CYT, NE) in 15ml tubes are precipitated with 4 times the volume of cold Ethanol at -20 °C overnight. Then they are centrifuged at 5000rpm at 4 °C for 30 min with acceleration of 9 and deceleration of 4 (Megafuge 16R Centrifuge, Thermo Scientific). Immediately after the centrifugation is finished the liquid is poured off. Whereas the liquid of the cell supernatant samples (SN) is kept for lipidomics and therefore stored at -20 °C, the liquid of the cytoplasm (CYT) and nuclear

extract (NE) fractions is discarded. The 15 ml tubes with the precipitated protein pellets are placed inverted on tissue paper for 10 min to drain off remaining liquid and then they are dried completely under vacuum in a desiccator. According to the size of the pellet a little amount of urea and an appropriate amount of sample buffer, which contains sodium dodecyl sulfate (SDS), are added and the samples are stored at -4 °C overnight. The next day the pellets are dissolved completely by resuspending several times and if necessary placing them in a sonic bath. If the whole pellet is dissolved the liquid is transferred to a 1.5 ml safe lock tube and the 15 ml tube is washed with 15 µl of sample buffer with SDS. All liquids are united in the 1.5 ml tube. If necessary the washing step is repeated to make sure that all protein is transferred to the 1.5 ml tube. After vortexing, the protein concentration is measured by Bradford assay and the samples are stored at -20 °C for further use.

3.3.2. Bradford assay

The protein concentration of all samples is measured by Bradford assay. Therefore 199 µl of LC-MS grade H_2O are pipetted in a well of a 96-well tissue culture plate (BD falcon) and 1 µl of the protein sample and 50 µl of Bradford reagent (Bio Rad) are added and mixed carefully by resuspending. A standard curve with bovine serum albumin (BSA, Roth) standards (0 µg/µl; 0.25 µg/µl; 0.5 µg/µl; 0.75 µg/µl; 1 µg/µl; 2 µg/µl; 3 µg/µl; 4 µg/µl and 5 µg/µl) is prepared in triplicates. The BSA stock solution contains no sample buffer, so 1 µl of sample buffer is added separately to the standards. If the concentration of the samples is exceeding the concentration range of the standard curve, the samples are diluted 1:1 with a blank solution (199 µl LC-MS grade H_2O + 1 µl sample buffer + 50 µl Bradford reagent). This step is repeated if necessary. The absorbance at 595 nm is measured by a microplate spectrometer (Multiscan GO, Thermo Scientific) and the data is exported to Microsoft Excel, where the protein concentrations of the samples are calculated. [Bradford, 1976]

3.3.3. Filter assisted in-solution digestion

For the filter assisted in-solution digest, the following reagents are prepared freshly before each digest. 500 mM ammonium bicarbonate (ABC) buffer is made of 197.5 mg ABC (Sigma Aldrich) and 5 ml H_2O in a 15 ml tube. This buffer is diluted (2 ml + 18 ml H_2O) in a 50 ml tube to create 50 mM ABC buffer. Aliquots of Dithiothreitol (DTT) and Iodoacetamide (IAA) are prepared on another day (41.96 g guanidinium hydrochloride (Sigma Aldrich)+ 274.5 mg DTT (Gerbu)+ or 549 mg IAA (Sigma Aldrich)+ to 49.5 ml with LC-MS grade H_2O in a 50 ml Falcon tube (Corning Scientific), 1100 µl aliquots in 1.5 ml tube (Eppendorf) and frozen at -20 °C). 120 µl of 500 mM ABC buffer are added per 1100 µl aliquot of DTT and IAA, they are thawed and vortexed. IAA is always kept in darkness, because it is sensitive to light. 10 kDa molecular weight cut-off filters (Nanosep® Centrifugal Devices, Pall Life Sciences) are placed in used and washed

tubes and the original tubes are saved for the enzyme reaction. 200 µl of the prepared DTT solution are pipetted in another 1.5 ml tube and the calculated volume for 20 µg of protein is added. The tubes are mixed with 1100 rpm for 30 min at 56 °C on a thermomixer (Thermomixer comfort, Eppendorf). In the meantime the filter is washed by adding 0.5 ml of LC-MS grade H_2O and centrifuging at 14000 g for 5 min (Centrifuge 5424, Eppendorf), so that all liquid passes through the filter. This step is planed so that the next solution is loaded on the filter right after centrifuging, so that the filter never dries out. After the incubation with DTT, the samples are cooled to 37 °C and they are loaded on the filter. The samples are centrifuged at 14000 g for 15 min and the flow-through is discarded. They are washed by adding 200 µl of 50 mM ABC buffer, centrifuging at 14000 g for 15 min and discarding the flow-through. 200 ml of the prepared IAA solution are added on the filter and the samples are incubated in the dark for another 30 min at 30 °C with 1100 rpm mixing on the thermomixer. After centrifuging at 14000 g for 15 min and removing the IAA flow-through, the samples are washed again by adding 200 µl of ABC buffer, centrifuging at 14000 g for 5 min and discarding the flow-through. The filter is placed in the original tube, 95 µl of cold 50 mM ABC buffer are added and the samples are mixed with 1100 rpm on the precooled thermomixer at 4 °C for 10 min. In the meantime the protease stock solution is prepared (20 µg Trypsin/Lys C mix + 200 µl Resuspension buffer, Promega) and placed on ice. 5 µl of protease stock solution are added and mixed with 1100 rpm at 4 °C for another 10 min. The samples are incubated at 37 °C in an incubator (Heratherm compact, Thermo Scientific) overnight (ca. 16.00 - 9.00). The next morning the samples are cooled at the thermomixer at 4 °C with mixing at 1100 rpm for 10 min. 45 µl of cold ABC buffer are added and mixed at 1100 rpm at 4 °C for another 10 min. After the addition of 5 µl protease stock solution the samples are mixed for 10 min at 4 °C with 1100 rpm at the thermomixer and then they are incubated for 4 h at 37 °C in the incubator. The samples are centrifuged at 14000 g for 10 min, 50 µl of LC-MS grade water are added to the filter and the samples are centrifuged again for 10 min at 14000 g. 50 µl of 0.5 %TFA are added to each sample and the peptides are resuspended by shaking on the thermomixer at RT for 10 min. After centrifuging at 14000 g for 10 min the collected flow-through contains the digested peptides. The samples are dried by vacuum centrifugation (miVac Duo Concentrator, GeneVac) at 40 °C and then they are stored at -20 °C. [Bileck *et al.*, 2014]

3.3.4. LC-MS/MS

The dried protein samples are dissolved in 5 µl 30 % FA containing 10 fM 4 synthetic standard peptides (Glu1-Fribrinopeptide B, EGVNDNEEGFFSAR; M28, TTPAVLDSDGSYFLYSK; HK0, VLETKSLYVR; HK1, VLETK(ε-AC)SLYVR, all from Sigma Aldrich) and 40 µl mobile phase A. All samples are measured by HPLC-MS/MS in technical duplicates. The HPLC is a Dionex Ultimate 3 000 HPLC system with a 75 µm x 2 cm

C18 Pepmap100 pre-column by Thermo Fisher Scientific and a 75 µm x 50 cm Pepmap100 analytical column by Thermo Fisher Scientific. Mobile phase A consists of 98 % H2O, 2 % ACN, and 0.1 % FA and mobile Phase B of 80 % ACN, 20 % H_2O and 0.1 % FA. A flow rate of 10 µl/min mobile Phase A is used for loading the peptides on the pre-column and they are eluted with flow rate of 300 µl/min mobile phase A to the analytical column. A 95 min gradient (8-40 % mobile phase B) for secreted proteins and a similar 235 min gradient for cytoplasmic and nuclear proteins is applied. 10 µl of each sample are injected and each sample is analyzed in technical duplicates. The HPLC is coupled to the nanospray ion source of a Q-Exactive Orbitrap mass spectrometer by Thermo Fisher Scientific. All scans are performed in negative ion mode. The m/z range for MS^1 is 400-1 400 with a resolution of 70 000 at m/z=200. The eight most abundant ions from secreted proteins and the twelve most abundant ions from cytoplasmic and nuclear proteins undergo HCD-fragmentation at 30 % normalized collision energy and are then measured in the orbitrap with a resolution of 17 500 at m/z=200. [Bileck *et al.*, 2014]

3.3.5. Data interpretation and bioinformatics

The protein identification and LFQ (label free quantification) is performed by using the MaxQuant software package (version1.5.2.8) which includes the Andromeda search engine. The statistical analysis and the data interpretation are carried out with the Perseus statistical analysis package (version 1.5.6.0). The measured peptides are searched against the Uniprot database for human proteins with a peptide mass tolerance of 25 ppm, an MS^2 match tolerance of 20 ppm and a maximum of two missed cleavages. Carbamidomethylation of cysteins is set as fixed modification and methionine oxidation and N-terminal protein acetylation as variable modifications. For secreted proteins, a minimum of one measured unique peptide was necessary to successfully identify a protein. A minimum of two measured peptides and one measured unique peptide was necessary to successfully identify a protein in cytoplasmic and nuclear samples. In all samples, a FDR (false discovery rate) of 0.01 for peptides was applied. [Bileck *et al.*, 2014]

In Perseus, the proteins are filtered for reversed sequences, potential contaminants and a minimum of three independent identifications per protein. All LFQ values are transformed by log2and a PCA (principal component analysis) is performed. An average of the two technical replicates is calculated, the different treatment groups are marked and the proteins are filtered for proteins, which are found in at least two out of three biological replicates. After filtering, missing values are replaced by values from normal distribution, so that a t-test can be performed. An annotation (GO molecular function, GO biological process, GO cellular component and KEGG pathway) is automatically added to all proteins and then a

two-sample t-test for two treatment groups (Con – LPS 20 h, LPS 20 h – (EGCG 44 h + LPS 20 h), Con – EGCG 44 h and EGCG 20 h – EGCG 44 h) with a permutation based multiparameter correction and with a false discovery rate (FDR) of 0.05 and a minimum fold change of 2.66 ($S_0 = 0.5$) is performed. A FDR of 0.05 means that out of all proteins claimed to be significant, a maximum of 5 % are not significant. The resulting significant and multiparameter corrected proteins are displayed as volcano plot. The Log2 of the LFQ intensity of the significant and multiparameter corrected proteins and the according standard deviation are displayed in graphs created with GraphPad Prism (version 6.07). [Bileck *et al.*, 2014]

The significant, multiparameter corrected upregulated proteins from all cell fractions are combined in one list and this list is submitted to DAVID web page for functional annotation. An overview of the upregulated KEGG pathways is shown in Table 6,all pathways with a Benjamini-score lower than 0.05 are considered significant. The same is done for significant and multiparameter corrected downregulated proteins, but there are too little proteins downregulated, so that no pathway is significantly represented. [Glynn *et al.*, 2003]

There are only 11 proteins significant and multiparameter corrected downregulated and 38 proteins significant and multiparameter corrected upregulated (Table 5), so a less strict significance test, a two-sample t-test for two treatment groups (Con – LPS 20 h, LPS 20 h – LPS 20 +EGCG 44 h, Con – EGCG 44 h and EGCG 20 h – EGCG 44 h) with a p-value of 0.05 and a minimum fold change of 2.66 ($S_0 = 0.5$) without multiparameter correction is performed. As before, the significant upregulated proteins from all cell fractions are merged to one list and the combined list is send to DAVID web page for functional annotation. The same is done for significant downregulated proteins and all pathways with a Benjamini-score lower than 0.05 are considered significant. [Glynn *et al.*, 2003]

4. Results and discussion

4.1. Cell culture

U937 cells grew without any difficulty in RPMI 1640 cell culture medium with 10 % FBS. When viewed in the microscope, they looked like single small, round dots. There were always cells in the foreground and in the background, because the cells were swimming in the cell culture media (Figure 5, A). After 24 h of incubation with PMA, the cells started to differentiate towards macrophages. This process was easily visible, because the cells started clotting together and they started to stick to the bottom of the cell culture flask (Figure 5, B). After another 72 h the differentiation was finished and there were nearly no single, swimming cells visible (Figure 5, C).

It was also checked, if there was a difference in the morphology of the cells in response to the treatment with LPS for 20 h and the treatment with EGCG for 44 h and LPS for 20 h compared to the control group. A comparison of Figure 5, D-F shows no visible difference.

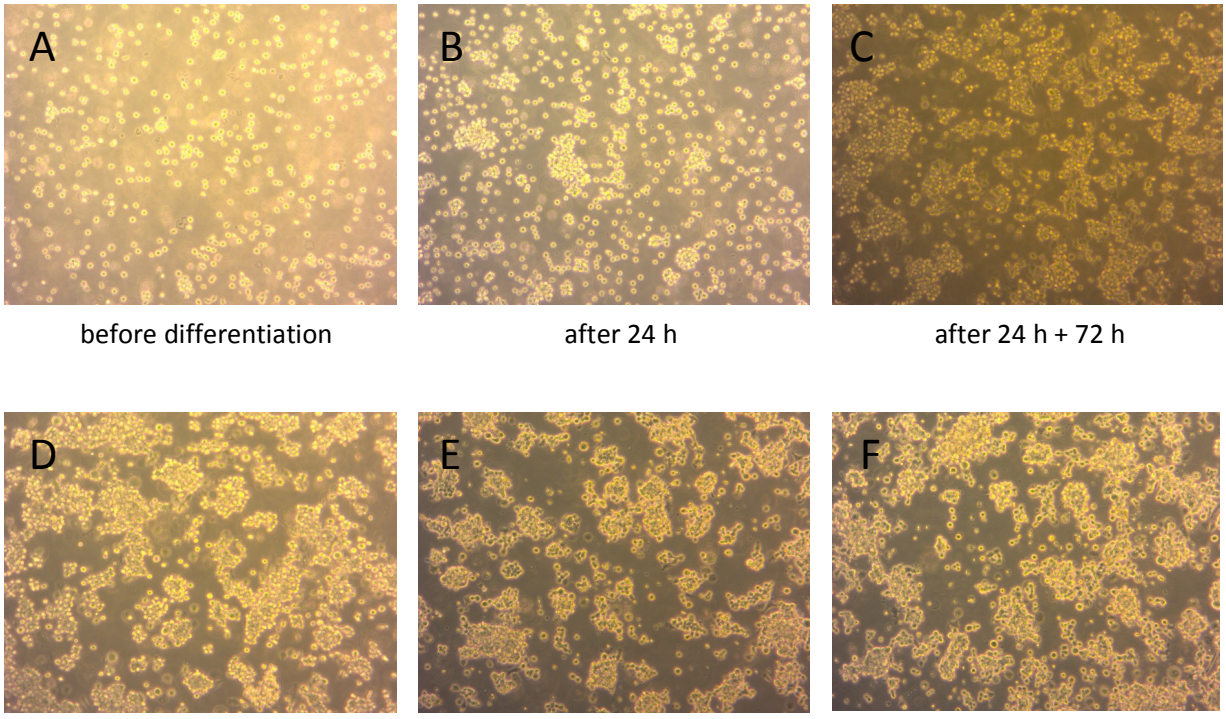

Figure 5: Pictures of U937 cells under the microscope. Comparison of different time points (A-C) and treatment conditions (D-F)

4.2. Lipidomics

The extraction and measurement has high reproducibility and reliability because the 3 internal standards are not regulated. This is obvious for 5S-HETE-d8 and PGF2a-d4, because all the data are normalized to them, as described above, but the third internal standard PGE2-d4 has a maximum variation of 1 % in all 4 comparisons (Table 2 and 3, Figure 6 and 7).

4.2.1. Con – LPS 20 h

The comparison of the control group and stimulated with LPS for 20 h shows that Tetranor 12-Hete, 12S-HHTrE, 11b-PGE2, PGD2, TBX1, TBX2, and a PGF2 like eicosanoid are significantly upregulated and PGA2, 8-iso-15-keto-PGE2, 13,14-dihydro-15-keto-PGD2 and 1a,1b-dihomo-PGF2aare newly expressed in samples stimulated with LPS for 20 h (Table 2). TBX2 and TBX1 show the highest fold change with 53.57 and 42.35 fold.

4.2.2. LPS 20 h– (EGCG 44 h +LPS 20 h)

The mediation of the LPS-stimulation with EGCG was successful, LPS 20 h compared with EGCG 44 h +LPS 20 h shows that 12S-HHTrE and TBX1, which were both upregulated with LPS 20 h are significantly downregulated by EGCG with a fold change of 0.56 and 0.43 (Table 2). All other Eicosanoids except the internal standards are downregulated as well, but these downregulations are not significant.

4.2.3. Con – EGCG 44 h and EGCG 20h – EGCG 44h

EGCG 44 h compared to control and EGCG 20 h compared to EGCG 44 h shows no significant regulations. The newly expressed Eicosanoids are not significant, because they variation from not detected to a maximum AUC of 0.0002 is too small (Table 3).

4.2.4. Summary

In summary, the secretion of eicosanoids like Tetranor 12-Hete, 12S-HHTrE, 11b-PGE2, PGD2, TBX1, TBX2, and a PGF2 like eicosanoids significantly increased by LPS and PGA2, 8-iso-15-keto-PGE2, 13,14-dihydro-15-keto-PGD2 and 1a,1b-dihomo-PGF2a were only detected in samples treated with LPS alone. This increase in the secretion of eicosanoids is partially modulated by EGCG,12S-HHTrE and TBX1 are significantly downregulated. EGCG alone over a maximum incubation time of 44 h seems to have no visible effect. A longer incubation period, according to normal green tea drinking habits, may change this. Furthermore due to the already described problem with the MS^2 scans, only 17 eicosanoids (3 internal standards) could be identified with high certainty. During the necessary RT-alignment of MS^1 scans several promising peaks had to be discarded, because their RT-time could be matched to several fitting RT-times on the created reference list.

Table 2: Results from the lipidomics part of this study. Comparison between Con - LPS 20 h and LPS 20 h - (EGCG 44 h + LPS 20 h). Newly expressed Eicosanoids marked with * are considered significant because the difference in peak area from 0 to their value (not shown) is bigger than the standard deviation. Compare with Figure 6 and 7.

			Con-LPS 20 h			LPS 20 h - (EGCG 44 h + LPS 20 h)		
Mass [m/z]	**R.T. [min]**	**Name**	**p-value**	**significant?**	**folds**	**p-value**	**significant?**	**folds**
265.1809	14.67	Tetranor 12-Hete	0.001400	yes	12.74	0.061235	no	0.74
279.1966	15.54	12S-HHTrE	0.000426	yes	9.64	0.031767	yes	0.56
295.2279	19.03	HODE like	0.635516	no	1.28	0.664560	no	0.80
311.2228	18.79	13S-Hpode	0.937438	no	0.94	0.785855	no	0.80
327.2781	21.02	5S-HETE-d8			1.00			1.00
333.2071	10.92	PGA2		yes*	newly expressed	0.386227	no	0.85
349.2021	7.93	8-iso-15-keto-PGE2		yes*	newly expressed	0.151793	no	0.78
351.2177	7.04	11b-PGE2	0.000175	yes	18.49	0.439413	no	0.84
351.2177	7.71	PGD2	0.002002	yes	13.43	0.280311	no	0.80
351.2177	9.62	13,14-dihydro-15-keto-PGD2		yes*	newly expressed	0.474336	no	0.85
353.2334	6.50	PGF2 like	0.000937	yes	5.56	0.678228	no	0.92
355.2428	7.00	PGE2-d4	0.762866	no	1.01	0.761326	no	1.01
355.2490	6.48	PGF1a	0.298235	no	1.37	0.334182	no	0.95
357.2585	6.46	PGF2a-d4			1.00			1.00
369.2283	5.64	TXB2	0.000105	yes	53.57	0.573234	no	0.90
371.2439	5.97	TXB1	0.000029	yes	42.35	0.016283	yes	0.43
381.2647	9.96	1a,1b-dihomo-PGF2a		yes*	newly expressed	0.772301	no	0.87

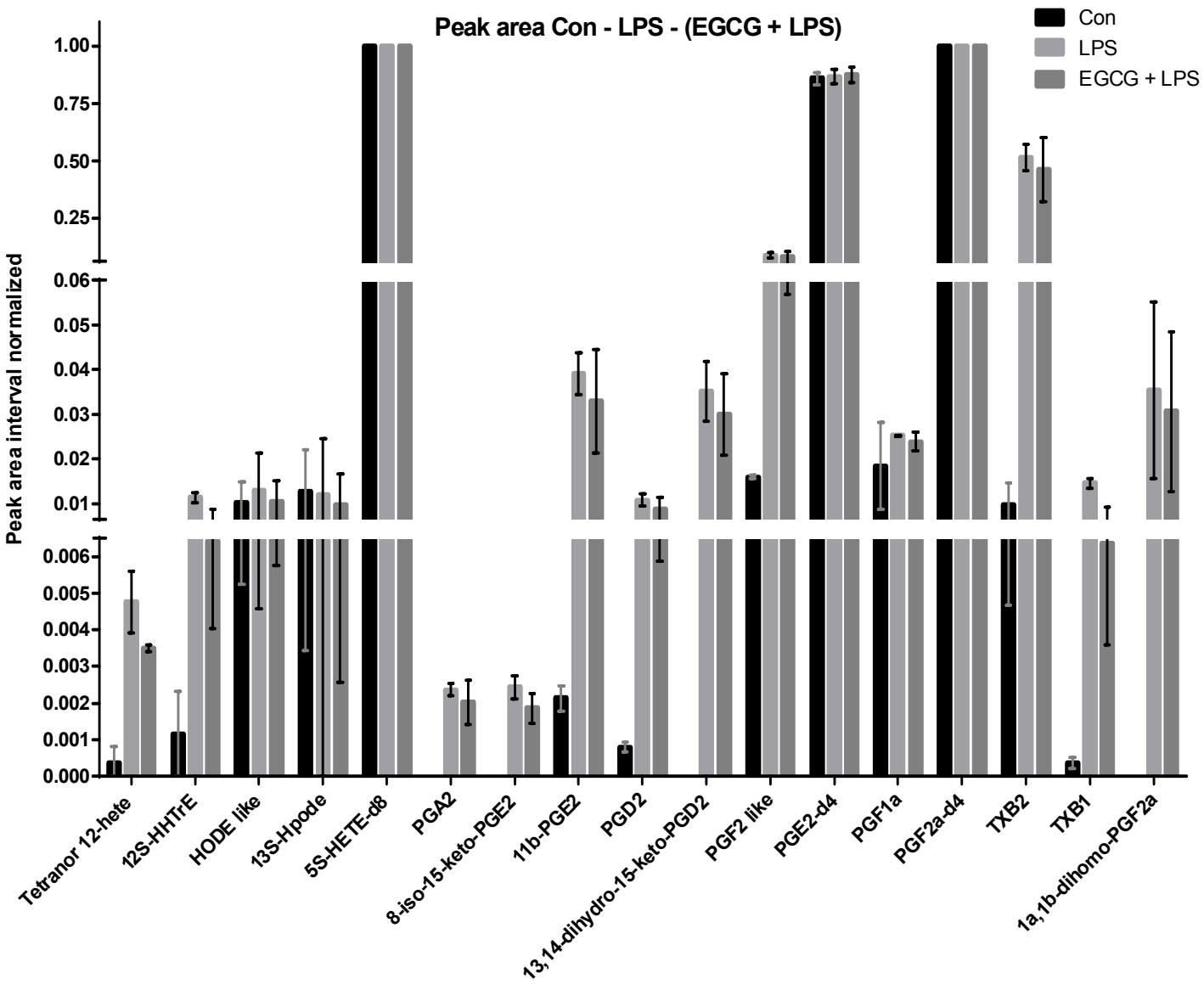

Figure 6: Diagram of the results from the lipidomics part of this study. Comparison between Con, LPS 20 h and (EGCG 44 h + LPS 20 h)

Table 3: Results from the lipidomics part of this study. Comparison between Con - EGCG 44 h and EGCG 20 h and EGCG 44 h). Newly expressed Eicosanoids marked with * are not considered significant because the difference in peak area from 0 to their value (not shown) is smaller than the average standard deviation. Compare with Figure 6 and 7.

			Con -EGCG 44h			EGCG 20h - EGCG 44h		
Mass [m/z]	**R.T. [min]**	**Name**	**p-value**	**significant?**	**folds**	**p-value**	**significant?**	**folds**
265.1809	14.67	Tetranor 12-Hete	0.847596	no	0.97	0.694439	no	0.68
279.1966	15.54	12S-HHTrE	0.492426	no	0.52	0.290032	no	0.32
295.2279	19.03	HODE like	0.965667	no	1.16	0.950959	no	1.08
311.2228	18.79	13S-Hpode	0.787207	no	0.95	0.554942	no	0.80
327.2781	21.02	5S-HETE-d8			1.00			1.00
333.2071	10.92	PGA2		no*	newly expressed			0.97
349.2021	7.93	8-iso-15-keto-PGE2		no*	newly expressed		no*	newly expressed
351.2177	7.04	11b-PGE2	0.802362	no	1.14	0.959136	no	1.04
351.2177	7.71	PGD2	0.629518	no	0.62	0.984057	no	0.88
351.2177	9.62	13,14-dihydro-15-keto-PGD2						
353.2334	6.50	PGF2 like	0.714769	no	0.99	0.968151	no	1.00
355.2428	7.00	PGE2-d4	0.951755	no	1.00	0.975630	no	1.00
355.2490	6.48	PGF1a	0.761810	no	0.69	0.639252	no	0.52
357.2585	6.46	PGF2a-d4			1.00			1.00
369.2283	5.64	TXB2	0.540579	no	1.18	0.714194	no	1.09
371.2439	5.97	TXB1	0.755188	no	0.80	0.924809	no	0.90
381.2647	9.96	1a,1b-dihomo-PGF2a		no*	newly expressed	0.780606	no	1.09

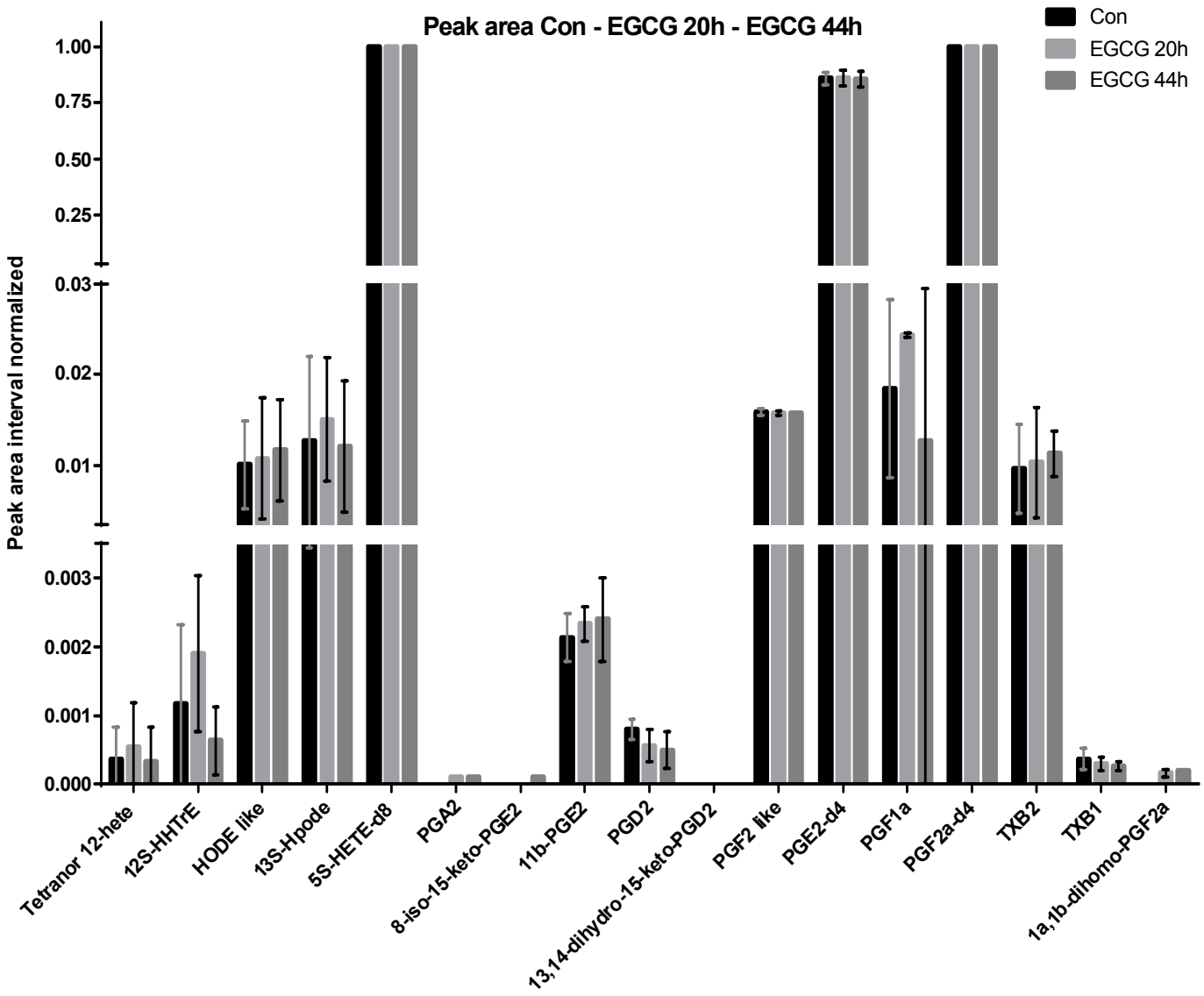

Figure 7: Diagram of the results from the lipidomics part of this study. Comparison between Con, EGCG 20 h and EGCG 44 h:

4.3. Proteomics

During the whole bioinformatics process, single interesting proteins can be checked manually via profile plot. Figure 8 shows the profile plot of interleukin 1β from all CYT-fractions. The relative amount of interleukin 1β is significantly increased in all samples, which were exposed to LPS and it looks like the prior treatment with EGCG has no influence on that increase.

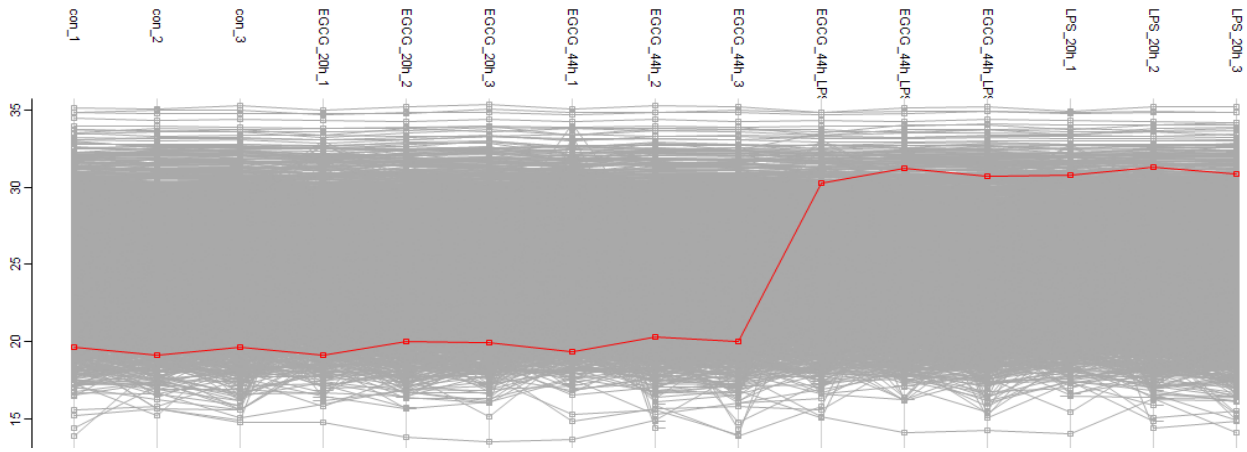

Figure 8: Exemplary profile plot of interleukin 1β from al CYT fractions under different treatment conditions

4.3.1. PCA

For each of the three fractions (SN, CYT and NE), a principal component analysis is performed. All three fractions show a similar pattern, for example, the result of the PCA for the NE samples is displayed in Figure 9.In total, there are four clusters. Samples, which were treated with LPS (LPS 20 h and EGCG 44 h + LPS 20 h) are separated from samples without LPS (Con, EGCG 20 h and EGCG 44 h). The biological replicate 1 separated from replicate 2 and 3. Although they show similar characteristics in matters of LPS and EGCG, this variation in the behavior of biological replicates may cause an increased uncertainty in the following analysis and statistics. All technical replicates (same symbol) are next to each other, so they are quite similar.

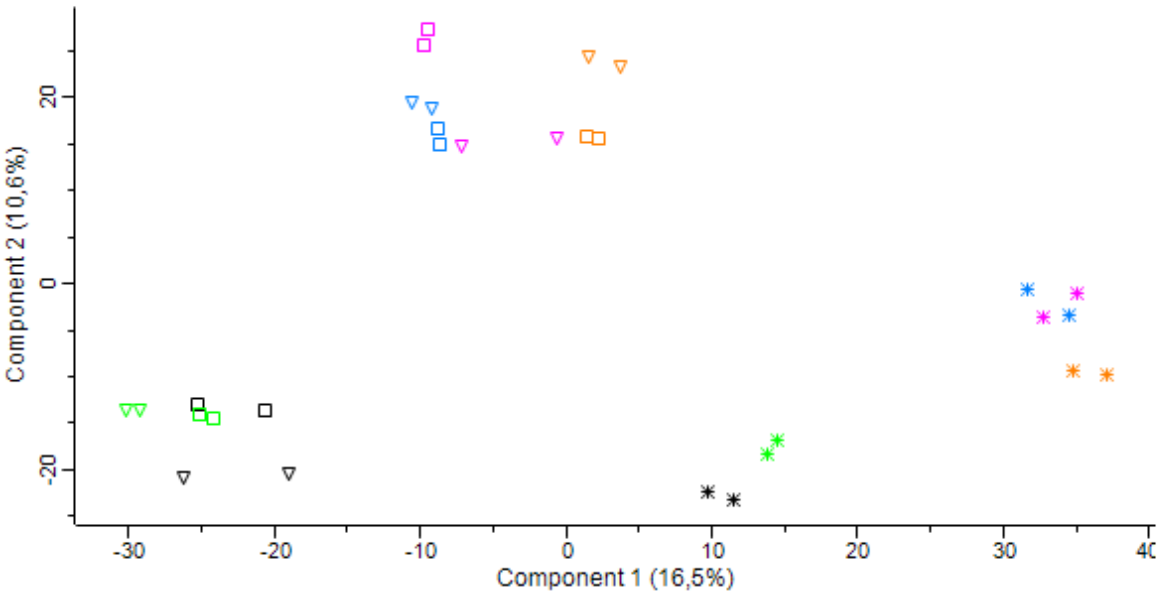

Figure 9: PCA of the NE samples

Table 4: Legend to Figure 9

Probe	Symbol color	Probe	Symbol type
Con	orange	1. biol. replication	✳
EGCG 20 h	blue	2. biol. replication	□
EGCG 44 h	purple	3. biol. replication	▽
LPS 20 h	black		
EGCG 44 h + LPS 20 h	green		

4.3.2. Significant, multiparameter corrected

A total of 4 two-sample t-tests (Con – LPS 20 h, LPS 20 h – (EGCG 44 h + LPS 20 h), Con – EGCG 44 h and EGCG 20 h – EGCG 44 h) for each subcellular fraction are performed and Proteins with a FDR of 0.05 and a minimum fold change of 2.66 (S_0 = 0.5) are considered significantly, multiparameter corrected regulated. Figure 10 shows an exemplary volcano plot of the comparison Con - LPS 20 h from the SN fraction. All proteins outside the black graphs are significantly, multiparameter corrected regulated. Table 5 shows an overview from all regulated proteins when compared Con with LPS 20 h. In all other comparisons (LPS 20 h – (EGCG 44 h + LPS 20 h), Con – EGCG 44 h and EGCG 20 h – EGCG 44 h) no proteins are significantly, multiparameter corrected regulated. Figure 11,12 and 13 show bar diagrams with error bars of all significant, multiparameter corrected regulated proteins.

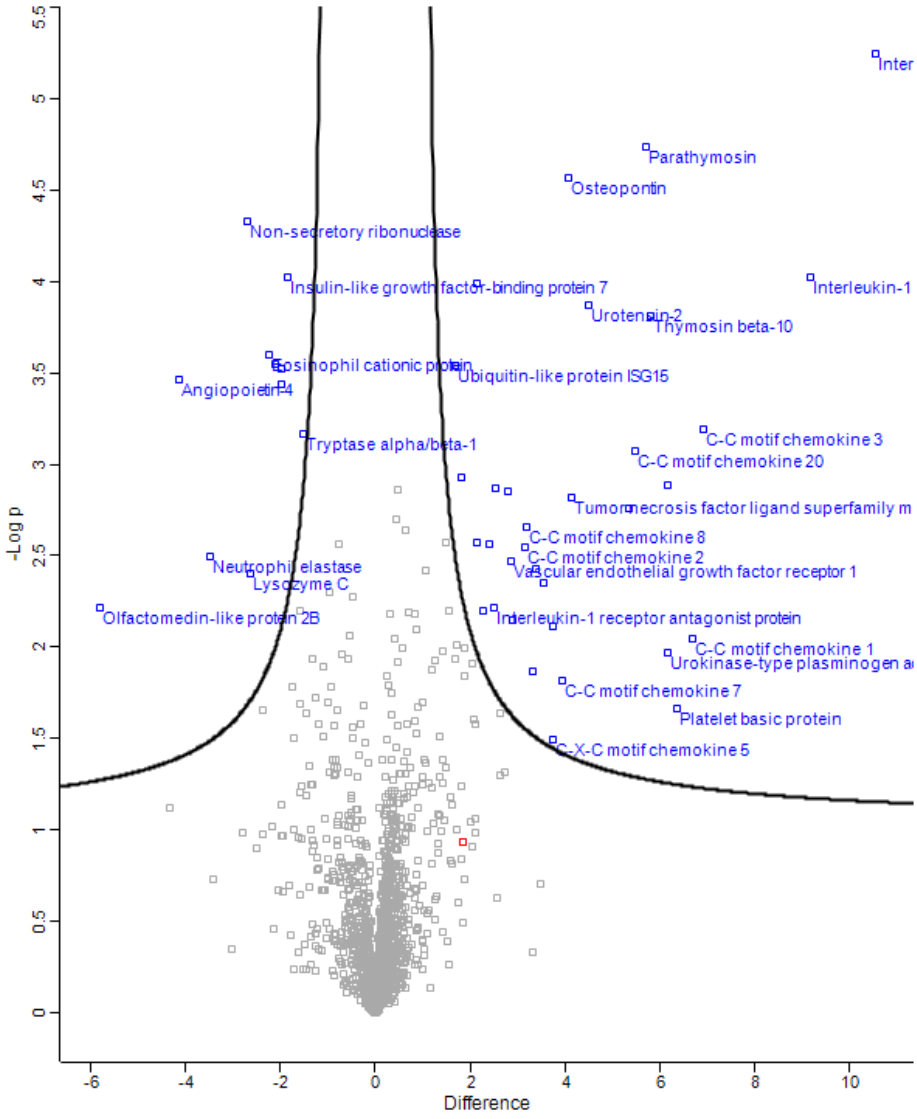

Figure 10: Exemplary volcano plot of the comparison Con - LPS 20 h from the SN fraction

Table 5: Overview of all significant, multiparameter corrected regulated proteins in the different fractions when compared Con with LPS 20 h

Con - LPS 20 h	**downreg.**	**upreg.**
SN	11	32
CYT	0	2
NE	0	4
total	11	38

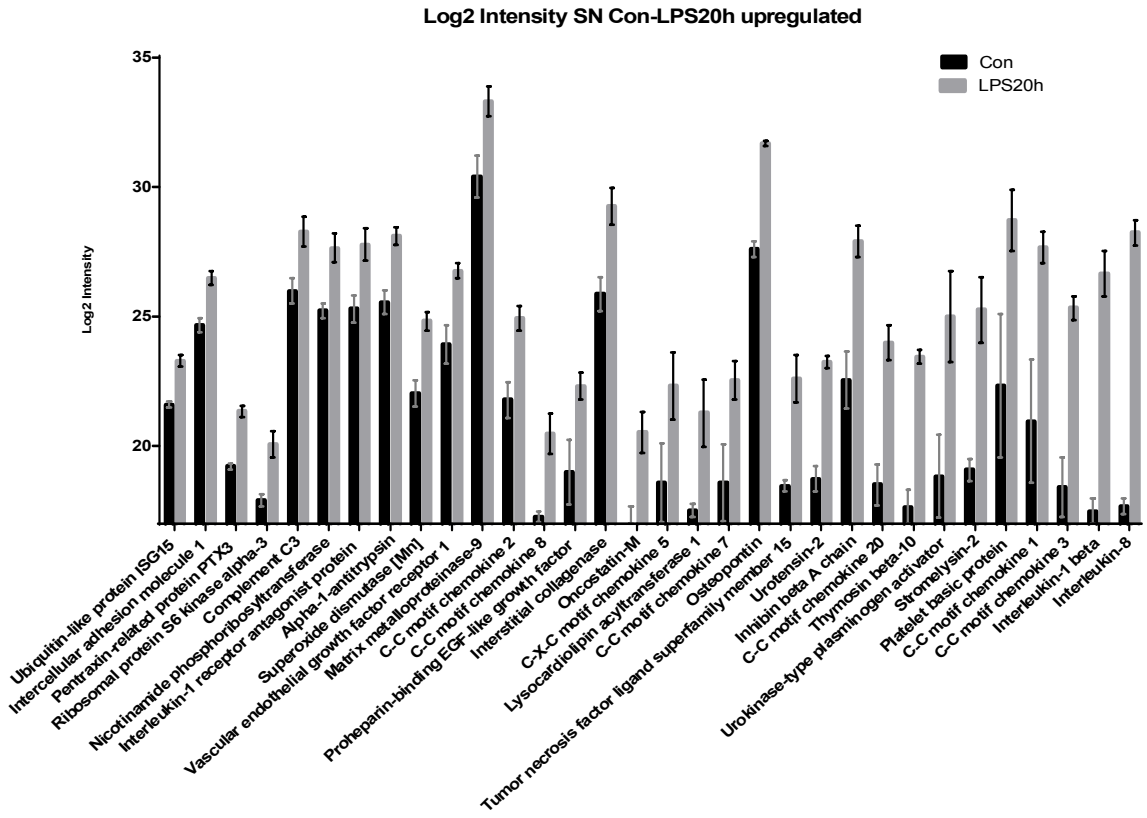

Figure 11: Significant, multiparameter corrected upregulated proteins in SN, comparison between Con and LPS 20 h

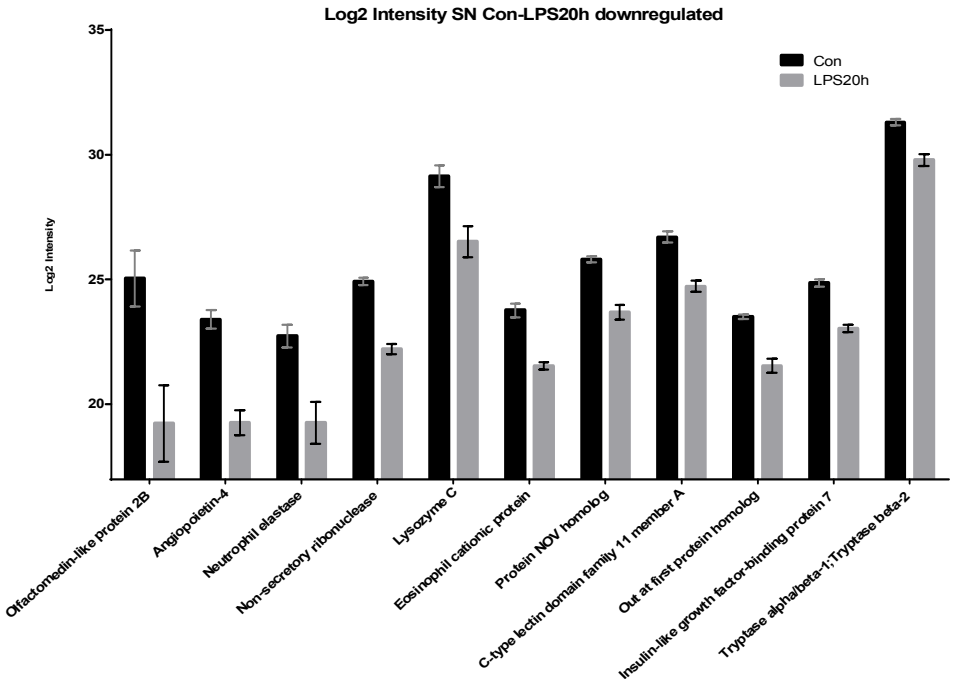

Figure 12: Significant, multiparameter corrected downregulated proteins in the SN fraction, comparison between Con and LPS 20 h

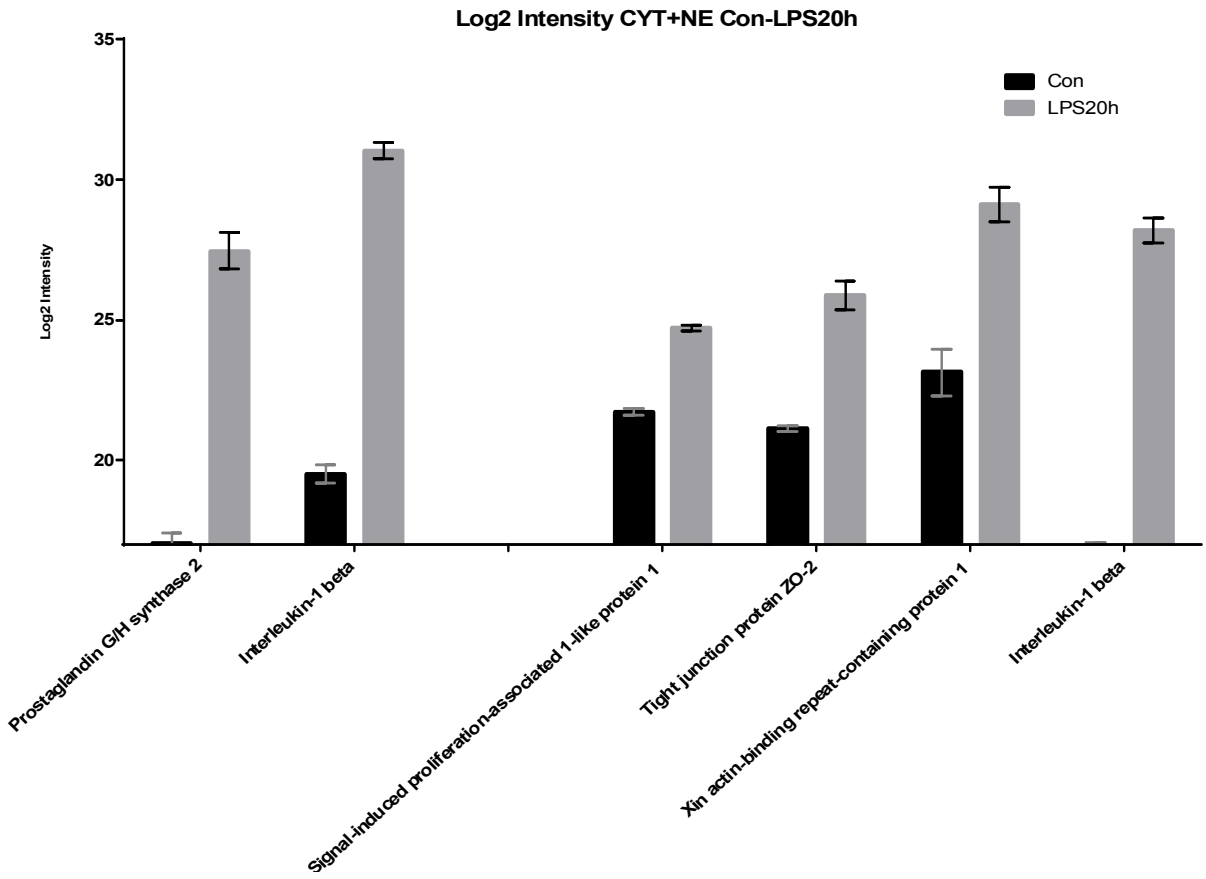

Figure 13: Significant, multiparameter corrected upregulated proteins in the CYT and NE fraction, comparison between Con and LPS 20 h

The results from the functional annotation of all significant, multiparameter corrected upregulated proteins are shown in Table 6. When compared Con with LPS 20 h, the strongest upregulated KEGG pathways are Chemokine signaling pathway, Rheumatoid arthritis Cytokine-cytokine receptor interaction and TNF signaling pathway. All these pathways are related to oxidative stress, inflammation and cancer. [Glynn, 2003]

Table 6: Results of the functional annotation with DAVID of all significant, multiparameter corrected upregulated proteins, comparison between Con and LPS 20 h

KEGG pathway upregulated *Con – LPS 20 h*	Count	%	p-value	Benjamini	significant?
Chemokine signaling pathway	11	28.9	4.30E-09	1.90E-07	yes
Rheumatoid arthritis	9	23.7	3.10E-09	2.80E-07	yes
Cytokine-cytokine receptor interaction	11	28.9	3.30E-08	9.80E-07	yes
TNF signaling pathway	6	15.8	9.60E-05	2.10E-03	yes
NF-kappa B signaling pathway	5	13.2	5.70E-04	1.00E-02	yes
Bladder cancer	4	10.5	7.80E-04	1.10E-02	yes
Chagas disease (American trypanosomiasis)	5	13.2	1.10E-03	1.40E-02	yes
Malaria	4	10.5	1.30E-03	1.40E-02	yes
Pertussis	4	10.5	4.40E-03	4.30E-02	yes
Toll-like receptor signaling pathway	4	10.5	1.20E-02	9.80E-02	no
NOD-like receptor signaling pathway	3	7.9	2.50E-02	1.70E-01	no
Legionellosis	3	7.9	2.40E-02	1.80E-01	no
Leishmaniasis	3	7.9	4.00E-02	2.10E-01	no
Influenza A	4	10.5	4.20E-02	2.10E-01	no
Transcriptional misregulation in cancer	4	10.5	3.90E-02	2.20E-01	no
Complement and coagulation cascades	3	7.9	3.80E-02	2.30E-01	no
Salmonella infection	3	7.9	5.30E-02	2.50E-01	no

Interleukin-1β is the strongest significant, multiparameter corrected upregulated protein in Con - LPS 20 h; it is upregulated in all three subcellular fractions.Interleukin-1β is upregulated 1496-fold in SN, 2967-fold in CYT and 2539-fold in NE. In SN, other pro-inflammatory cytokines and chemokines like Interleukin-8 (577-fold), C-C modified chemokine 3 (120-fold) and C-C modified chemokine 1 (104-fold) are upregulated as well.

Furthermore it is interesting, that Prostaglandin G/H synthase 2 (=COX 2) is strongly upregulated (1354-fold) in the CYT fraction of Con - LPS 20 h. COX 2 is responsible for the synthesis of eicosanoids or precursor of eicosanoids. This increase in the amount of COX 2 fits well to the increase in the amount of eicosanoids which was observed in the Lipidomics part of this study.

In summary, the production of pro inflammatory cytokines and chemokines is increased significantly, multiparameter corrected by LPS. The strongest effect was found in SN, but it is also visible in CYT and NE samples. The functional annotation by DAVID shows that pathways related to oxidative stress, inflammation and cancer are significantly upregulated by LPS. There is no significant, multiparameter corrected modulation of this increase by EGCG and no effect of EGCG alone visible.

4.3.3. Significant

A less strict t-test without multiparameter correction was performed because only eleven proteins were significant and multiparameter corrected downregulated and 38 proteins significant and multiparameter corrected upregulated.

A total of 4 two-sample t-tests (Con – LPS 20 h, LPS 20 h – (EGCG 44 h + LPS 20 h), Con – EGCG 44 h and EGCG 20 h – EGCG 44 h) for each subcellular fraction are performed and Proteins with a p-value of 0.05 and a minimum fold change of 2.66 (S_0 = 0.5) are considered significantly regulated. Table 7 shows an overview from all regulated proteins for all two-sample t-tests.

Table 7: Overview of all significant regulated proteins in the different fractions

Con - LPS 20 h	**downreg.**	**upreg.**	***LPS 20 h - EGCG+LPS***	**downreg.**	**upreg.**
SN	39	69	**SN**	7	1
CYT	112	113	**CYT**	27	17
NE	144	168	**NE**	25	23
total	295	350	**total**	59	41
Con - EGCG 44 h	**downreg.**	**upreg.**	***EGCG20 h - EGCG 44 h***	**downreg.**	**upreg.**
SN	2	6	**SN**	8	4
CYT	58	43	**CYT**	45	56
NE	25	35	**NE**	24	27
total	85	84	**total**	77	87

The results from the functional annotation with DAVID are shown in Table 8 and Table 9. Only the comparison between Con and LPS 20 h is shown, because in all other comparisons (LPS 20 h – (EGCG 44 h + LPS 20 h), Con – EGCG 44 h and EGCG 20 h – EGCG 44 h), no pathway is significantly regulated. When compared Con with LPS 20 h, the strongest upregulated KEGG pathways over all fractions are TNF signaling pathway and NF-kappa B signaling pathway and the strongest downregulated pathways are Lysosome and Biosynthesis of antibiotics. All significant upregulated pathways are related to oxidative

stress, inflammation and cancer. The significant downregulated KEGG-pathways Lysosome and Glycosaminoglycan degradation are involved in the decomposition of biomolecules, whereas Biosynthesis of antibiotics and mTOR signaling pathway are related to the body's own defense system against bacteria. LPS plays an important role in the defense system of Gram-negative bacteria, so it makes sense that LPS would try to shut down pathways, which are responsible for the defense of cells against bacteria. [Glynn *et al.*, 2003; Martich *et al.*, 1993]

Table 8: Results of the functional annotation with DAVID of all significant upregulated proteins, comparison between Con and LPS 20 h

KEGG pathway downregulated Con – LPS 20 h	**Count**	**%**	**p-value**	**Benjamini**	**significant?**
Lysosome	11	3.7	1.40E-04	2.80E-02	yes
Biosynthesis of antibiotics	14	4.7	3.00E-04	3.00E-02	yes
Glycosaminoglycan degradation	5	1.7	4.70E-04	3.10E-02	yes
mTOR signaling pathway	7	2.3	9.80E-04	4.90E-02	yes
Other glycan degradation	4	1.3	5.10E-03	1.90E-01	no
Carbon metabolism	8	2.7	7.20E-03	2.20E-01	no
Leukocyte transendothelial migration	8	2.7	9.10E-03	2.30E-01	no
Biosynthesis of amino acids	6	2	1.60E-02	3.30E-01	no
Fatty acid degradation	4	1.3	5.70E-02	7.30E-01	no
Amino sugar and nucleotide sugar metabolism	4	1.3	7.00E-02	7.70E-01	no
Glutathione metabolism	4	1.3	8.10E-02	7.90E-01	no
Epstein-Barr virus infection	8	2.7	8.50E-02	7.80E-01	no
Metabolic pathways	32	10.7	9.20E-02	7.80E-01	no
Ribosome biogenesis in eukaryotes	5	1.7	9.60E-02	7.70E-01	no
NF-kappa B signaling pathway	5	1.7	9.60E-02	7.70E-01	no

Table 9: Results of the functional annotation with DAVID of all significant downregulated proteins, comparison between Con and LPS 20 h

KEGG pathway upregulated Con – LPS 20 h	Count	%	p-value	Benjamini	significant?
TNF signaling pathway	19	6	8.00E-11	1.60E-08	yes
NF-kappa B signaling pathway	15	4.7	2.30E-08	2.30E-06	yes
Herpes simplex infection	20	6.3	1.10E-07	7.40E-06	yes
Viral carcinogenesis	20	6.3	6.60E-07	3.30E-05	yes
Chemokine signaling pathway	19	6	7.00E-07	2.80E-05	yes
Epstein-Barr virus infection	19	6	9.60E-07	3.20E-05	yes
Influenza A	18	5.7	1.30E-06	3.60E-05	yes
Rheumatoid arthritis	13	4.1	1.50E-06	3.80E-05	yes
Osteoclast differentiation	15	4.7	4.00E-06	9.00E-05	yes
Toll-like receptor signaling pathway	13	4.1	1.10E-05	2.20E-04	yes
Transcriptional misregulation in cancer	16	5.1	1.70E-05	3.00E-04	yes
Amyotrophic lateral sclerosis (ALS)	9	2.8	2.80E-05	4.60E-04	yes
Chagas disease (American trypanosomiasis)	12	3.8	5.00E-05	7.70E-04	yes
Bladder cancer	8	2.5	5.80E-05	8.30E-04	yes
Cytokine-cytokine receptor interaction	17	5.4	1.80E-04	2.40E-03	yes
Epithelial cell signaling in Helicobacter pylori infection	9	2.8	2.30E-04	2.90E-03	yes
Legionellosis	8	2.5	3.50E-04	4.10E-03	yes
NOD-like receptor signaling pathway	8	2.5	3.90E-04	4.30E-03	yes
Amoebiasis	10	3.2	1.20E-03	1.30E-02	yes
Malaria	7	2.2	1.30E-03	1.30E-02	yes
Proteoglycans in cancer	14	4.4	1.40E-03	1.30E-02	yes
Fc epsilon RI signaling pathway	8	2.5	1.40E-03	1.30E-02	yes
Leishmaniasis	8	2.5	1.80E-03	1.60E-02	yes
Pertussis	8	2.5	2.50E-03	2.10E-02	yes
Hepatitis B	11	3.5	3.20E-03	2.50E-02	yes
Salmonella infection	8	2.5	4.40E-03	3.40E-02	yes
Measles	10	3.2	5.80E-03	4.20E-02	yes

HTLV-I infection	14	4.4	1.10E-02	7.80E-02	no
Pathways in cancer	18	5.7	1.80E-02	1.20E-01	no
MAPK signaling pathway	13	4.1	2.50E-02	1.60E-01	no
Complement and coagulation cascades	6	1.9	2.80E-02	1.70E-01	no
RIG-I-like receptor signaling pathway	6	1.9	3.00E-02	1.70E-01	no
Adherens junction	6	1.9	3.10E-02	1.80E-01	no
Tuberculosis	10	3.2	3.20E-02	1.80E-01	no
Phagosome	9	2.8	3.70E-02	1.90E-01	no
Hepatitis C	8	2.5	4.80E-02	2.40E-01	no
Micro RNAs in cancer	13	4.1	5.20E-02	2.50E-01	no
Fc gamma R-mediated phagocytosis	6	1.9	5.80E-02	2.70E-01	no
Inflammatory bowel disease (IBD)	5	1.6	7.50E-02	3.30E-01	no
Shigellosis	5	1.6	7.50E-02	3.30E-01	no
GnRH signaling pathway	6	1.9	7.60E-02	3.30E-01	no
B cell receptor signaling pathway	5	1.6	9.20E-02	3.80E-01	no
HIF-1 signaling pathway	6	1.9	9.70E-02	3.90E-01	no

Of course, as described above, interleukin-1β is the strongest significant upregulated protein in Con - LPS 20 h. It is upregulated 1496-fold in SN, 2967-fold in CYT and 2539-fold in NE. In SN, other pro-inflammatory cytokines and chemokines like interleukin-8 (577-fold), C-C modified chemokine 3 (120-fold) and C-C modified chemokine 1 (104-fold) are upregulated as well. Also prostaglandin G/H synthase 2 (=COX 2) is 1354-fold upregulated in the CYT fraction of Con - LPS 20 h. But furthermore in the SN fraction, angiopoietin-4 is downregulated 17.6-fold and neutrophil elastase is downregulated 11.3-fold by LPS. Angiopoietin-4 promotes endothelial cell survival, migration and angiogenesis. Neutrophil elastase modifies the functions of natural killer cells and monocytes and among others it can negative regulate the biosynthesis of chemokines. Cyclin-H is 29-fold downregulated by LPS in the CYT-fraction and is involved in cell cycle control and RNA-transcription. [Eki *et al.,* 1997; Lee *et al*., 2004; Nemoto *et al.*, 2002]

When compared LPS 20 h to EGCG 44 h + LPS 20 h, intelectin-1, which binds to glycans of bacteria, is upregulated 13.3-fold by EGCG in CYT. In NE, Sn1-specific diacylglycerol lipase alpha, which plays a role in arachidonic acid metabolic process and neurotransmitter biosynthetic process, is downregulated 14.5-fold. The comparison between Con and EGCG 20 h shows, that EGCG alone upregulates urotensin-2, a

potent vasoconstrictor, 23.4-fold in SN and downregulates sodium channel protein type 2 subunit alpha 66,5-fold in CYT. EGCG 20 h compared to EGCG 44 h shows, that caspase-1, which is important for defense against pathogens and can promote apoptosis, is downregulated 19.1-fold in NE and MAP kinase-interacting serine/threonine-protein kinase 1, a protein which may plays a role in response to environmental stress and cytokines, is upregulated 6.7-fold in NE. [Douglas *et* Ohlstein, 2001; Fuchs *et* Steller, 2011; Fukunaga *et* Hunter, 1997; NCBI gene database, 2017; Lee *et al.*, 2015; Tanimura *et al.,* 2010; Wesener *et al.,* 2015]

In summary, the production of pro inflammatory cytokines and chemokines is increased significantly by LPS. Functional annotation by DAVID shows that pathways related to oxidative stress and inflammation are significantly upregulated by LPS and some housekeeping pathways are significantly downregulated by LPS. Some proteins are significantly regulated by EGCG, but their regulation is not as strong as with LPS and functional annotation by DAVID shows that no KEGG pathway is significantly regulated.

4.3.4. Summary

To give a resume over the proteomics results, the PCA indicates that there is a big difference between samples with LPS and without LPS. An influence from EGCG is not visible. A detailed observation of the regulated proteins and the analysis with DAVID seems to prove this hypothesis. There is a strong regulation of interleukin-1β and other cytokines and chemokines by LPS, but for EGCG there are only weak regulations visible, and these effects can only be observed with the reduced reliability of omitting multiparameter correction. The functional annotation with DAVID shows exactly the same, pathways related with oxidative stress and inflammation are significant upregulated with LPS, with EGCG no pathway is significantly regulated.

When compared significant with multiparameter correction with significant without multiparameter correction, both results are nearly the same, without multiparameter correction shows a little more details.

4.4. The whole picture

When putting proteomics and lipidomics results together, the induction of inflammation with LPS was successful. The secretion of six eicosanoids significantly increased and four eicosanoids were newly expressed. According to that pro inflammatory cytokines and chemokines likeinterleukin-1β, interleukin-8, C-C modified chemokine 3 and C-C modified chemokine 1 are strongly upregulated. The

functional annotation by DAVID shows that pathways related to oxidative stress, inflammation and cancer are significantly upregulated and some housekeeping pathways are significantly downregulated by LPS. Furthermore prostaglandin G/H synthase 2 (=COX 2), a protein which is necessary for the synthesis of eicosanoids, is strongly upregulated. This fits well to the increased release of eicosanoids.

The modulation of the LPS induced inflammation by EGCG was only partially successful. In the lipidomics part of this study, only two eicosanoids, 12S-HHTrE and TXB, which were both upregulated with LPS, are significantly downregulated by EGCG, but 8-iso-15-keto-PGE2 and 11b-PGE2, two eicosanoids that are formed from arachidonic acid during the attack of free radicals, are not significantly regulated. It was expected, that eicosanoids created by non-enzymatic oxidation by ROS would be decreased significantly, because EGCG possesses radical scavenging properties due to its polyphenolic nature. On the proteomics side, single proteins which may help the body to deal with LPS and improve the defense against bacteria, are regulated by EGCG, but the functional annotation by DAVID shows that no pathway is significantly regulated.

EGCG alone, no matter if applied for 20 h or 44 h, showed no noteworthy significant effect, on lipidomics side as well as on proteomics side. The regulation of single proteins is registered, but this is no significant effect and an interpretation without further investigation would be meaningless.

The reason why EGCG shows only a weak to no effect may be the short incubation time. A maximum of 44 h incubation may be far too low to show as strong effects, as described in various empiric studies. Most of these studies have incubation times of several weeks to resemble normal tea drinking habits. In that case EGCG can accumulate and the whole system can adept and react to EGCG. To receive beneficial results in the fight against cancer, it seems to be necessary to not only activate or inhibit single pathways temporary, rather it is necessary to shift the whole network of pathways in cell signaling in a favorable direction. This influence can only be achieved with multiple doses over a longer time period. Phytochemicals are considered to affect human health in general and cell signaling in detail, but normally only if they are consumed regularly over a longer period of time. It would be beneficial to repeat this study with a drastically increased incubation time of EGCG to get better results. The low concentration of EGCG of 1 µM was chosen on purpose, to resemble a concentration, which could be achieved in human blood plasma due to normal tea drinking habits. More dramatic effects would have been visible with a drastically increased concentration of EGCG, as shown in other studies. [De Bacquer *et al.,*2006; Flores-Pérez *et al.*,2016; Nakagawa *et al.*, 1997; Srinivasan *et al.,* 2004]

5. Summary and conclusion

Cancer is next to coronary heart diseases the second leading cause of death worldwide. In contrary to other diseases, although mainly caused by environmental factors, a clear single elicitor of cancer is often not visible. Carcinogenesis, the process of developing cancer, is a long term multistage process, where each step can be reversed, or at least slowed down or nearly frozen the progression. Furthermore the carcinogenesis can always be terminated when the affected cells die. The multistage process of carcinogenesis can be influenced in both directions by many factors, but two closely related factors are oxidative stress and inflammation. However these two are not independent, they are influenced by mainly environmental factors, whereof one of the most important factors is diet. Especially phytochemicals are considered to have a positive and stabilizing effect on human health. Many studies prove that green tea and especially EGCG, which is next to caffeine one of the most effective phytochemicals in green tea, has a positive effect on human health, stabilize cellular pathways, help the body to keep control over cellular regulations and cell cycle control and therefore repel cancer. Although lots of studies prove the general effect of green tea describing different affected pathways, a general understanding of the whole pathway-network is still missing. So the aim of this study was to investigate the influence of EGCG on LPS stimulated U937 monocytes and measure as many relevant factors as possible simultaneously. Therefore lipidomics and proteomics of the same cells were combined and for the analysis of proteins, the subcellular fractions supernatant, cytoplasm and nucleus were separated. In the lipidomics part of the study only eicosanoids, which are important signal molecules, where measured, whereas in the proteomics part of the study the whole proteome in all three subcellular fractions was measured. All measurements were done with HPLC-MS/MS and the resulting data was analyzed with different bioinformatics tools.

When summing up all the results, the data demonstrates that the treatment of U937 monocytes with LPS has a profound effect. On the Lipidomics side the amount of pro inflammatory eicosanoids was found increased and on the proteomics side the level of pro inflammatory cytokines and chemokines was also increased in all three subcellular fractions. A functional annotation with DAVID shows that pathways related to oxidative stress, cancer and inflammation were significantly upregulated, whereas some "housekeeping" pathways were significantly downregulated by LPS. The influence of EGCG was not that clearly visible. When applied both, LPS and EGCG, EGCG managed to modulate the LPS induced upregulation of several eicosanoids, but in contrast to EGCG´s radical scavenging properties eicosanoids, created non-enzymatically during the attack of ROS, were not significantly regulated. Even some proteins, which may help the cells to deal with LPS, where upregulated by EGCG, but in total, no pathway

was significantly regulated when compared to LPS alone. When applying EGCG alone, regardless of an incubation time of 20 h or 44 h, neither on the lipidomics, nor on the proteomics side there was no significant effect detectable.

The low or almost inexistent effect of EGCG may originate in the short incubation time of EGCG. In further studies longer incubation times of EGCG should be taken into consideration, which resemble normal tea drinking habits. The chosen EGCG concentration on 1 µM seems to be adequate, as it resembles concentrations, which could be found in human blood upon normal tea drinking habits. Also the approach to combine lipidomics and proteomics to get a grip on the whole system seems to be beneficial, as oxidative stress, inflammation and cancer are processes, which influence the whole system and cannot be described and fully understood if reduced to only single pathways or molecules. It is already known that EGCG can alter gene expression, so it would be interesting to include also transcriptomics to the array of measurements.

In summary, the present study has achieved important objectives. The stimulation of inflammation in U937 monocytes by LPS was successful and caused a large number of significant regulatory events, while EGCG showed a rather subtle modulatory effect. The multi -omics approach was technically highly successful and produced complementary information. This might serve as an inspiration for others in future studies to consider and try to understand the whole picture of a cells signaling network. Because only with this broad view there may be a chance to really understand and one day even "cure" cancer and consequently save millions of lives.

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