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„Disease associated protein alteration of platelets of melanoma patients is associated with characteristic eicosanoid patterns in the releasate“

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Abbreviations

The systematic names of the found eicosanoids and the corresponding abbreviations are listed in table 4 and 5.

12-LOX	Arachidonate 12-lipoxygenase
15-LOX	Arachidonate 15-lipoxygenase
5-LOX	Arachidonate 5-lipoxygenase
AA	arachidonic acid
ACN	acetonitrile
BLT	Leukotriene B4 receptor
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CID	collision induce dissociation
COX	cyclooxygenase
cPLA2	cytosolic phospholipase A2
CYP	cytochrome P450
DNA	deoxyribonucleic acid
ESI	electron spray ionisation
FA	formic acid
FCS	fetal calf serum
FT	Fourier transformation
FWHM	full width at half maximum
GPCR	G protein-coupled receptor
HEPE	Hydroxy/hydroperoxyeicosapentaenoic acids
HETE	Hydroxy/hydroperoxyeicosatetraenoic acids
HHTrE	Hydroxy/hydroperoxyeicosatrienoic acids
HPLC	high-performance liquid chromatography
LMSD	LIPID MAPS Structure database
LOX	lipoxygenase
LT	leukotriene
MALDI	Matrix-Assisted Laser Desorption/Ionisation
MeOH	Methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
NK	natural killer cells
NLS	neutral-loss scan

PAD	peripheral arterial diseases
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PGHS	Prostaglandin G/H synthase
PIS	Precursor-ion scan
PRP	platelet rich plasma
PUFA	polyunsaturated fatty acid
QqQ	Triple quadrupole mass spectrometer
RNA	ribonucleic acid
SDS	sodiumdodecyl sulfate
SPE	solid-phase extraction
SRM	selected reaction monitoring
TOF	time-of-flight
TX	Thromboxane
VEGF	vascular endothelial growth factor
XIC	Extracted-ion chromatogram

1. Introduction

Human blood has always been a great source for analytical approaches for several diseases. Many of its compartments are today targeted for standardized clinical tests in order to detect specific biomarkers or other disease related indicators [1]. The composition of blood and its properties as an indicator for the whole system is nowadays a big analytical field. With the latest analytical methods like high-performance liquid chromatography (HPLC) and mass spectrometry (MS) it is possible to create high-throughput methods for proteomic or metabolomics approaches in a rather short time [2]. Blood serum as one of the most important compartments for bioanalytics is used for many shotgun and targeted approaches [1].

Blood platelets as one of the key players for inflammation and homeostasis are another target compartment of blood that can be used for analyses. Many diseases, especially when they are a matter of blood, are proven to be correlated with platelets [3]. While the link to vascular diseases, inflammation or thrombosis is somewhat apparent, the correlation to tumor growth and especially tumor progression is not that obvious. Nevertheless, it has been shown that the formation of metastases is promoted through the presence of platelets [4]. Furthermore, not only do blood platelets affect tumor metastasis but the platelets also seem to be affected by cancer diseases [5]. It was also assumed that the platelets themselves are entering a somehow exhausted state in some malignant tumor patients [6].

Eicosanoids are one of the small signalling molecules that are produced during activation of blood platelets but are also present in all other cell types [7]. Their effects show in a wide range but especially during inflammation a great amount of eicosanoids are produced to transduce the signal. But also for homeostatic actions, aggregation or penetrating the vascular cell wall eicosanoids are essential for platelet activity [3]. Beside the proteins these metabolites can be a great target for the analyses of platelet activation.

With the advanced technologies in HPLC and mass spectrometry, eicosanoids, along with other lipids and metabolites, are better accessible than ever before. With regard to the analytical process only, it is possible to analyse a complex biological matrix qualitatively and quantitatively within short time [8]. This process shows a high accuracy and reproducibility and is therefore a suitable technique for the fundamental analysis of biological samples [9,8].

In the centre of this work is the analysis of blood platelets derived from patients diagnosed with metastatic melanoma and the comparison with a healthy donor. The aim was to show a difference in the releasate after the activation with ionomycin. Therefore the blood platelets of four patients and one healthy donor were derived and activated with the Ca^{2+}-ionophore

ionomycin. The supernatant of the platelets was purified for eicosanoids and analysed with a HPLC-MS/MS system. The obtained data was analysed and quantified manually. Afterwards, the results of the healthy donors and patients were compared to show the difference in the activity of the obtained platelets. The main purpose of this work was therefore to offer an analytical tool which clearly reveals the biological differences that arise in the activity of platelets when derived from patients with metastatic melanoma compared to healthy platelets.

2. Platelets, melanoma and other diseases

2.1. Melanoma

Melanoma is a form of skin cancer originated from melanocytes which tends to be very aggressive. These cells are normally in the basal layer of the epidermis where they synthesise the pigment melanin and therefore induce the darkening of the skin when exposed to sunlight [10]. This normal exposure to UV light can mutate the cells and furthermore cause the formation of skin cancer. Studies show that the incidence of melanoma is highly related to environmental factors as well as genetic disposition [11]. The time of sun exposure as well as unnatural contact to UV light for example through the usage of sunbeds are correlated with a higher incidence of melanoma formation. Furthermore, there is a correlation between genetic factors and skin cancer. Humans with pale white skin for example show a higher probability to be diagnosed with melanoma [10]. As with most other cancers a correlation is seen for patients whose relatives were diagnosed with melanoma as well as older people. These show a significantly higher likelihood to develop melanoma [11,10].

When diagnosed in an early stage the 5-year survival rate can be up to 90% when the tumor is removed surgically [12]. That is why a lot of research is done on methods to recognise the development of melanoma. On the other hand, when diagnosed in a later, malignant stage especially when the tumor has already started to form metastases the chances for a cure decrease drastically. Classical therapeutic approaches like chemotherapy have been shown to show little to no effect and do not increase the overall survival rate of the patients significantly [12].

Newer strategies like immunotherapy and targeted therapy seem very promising and are currently researched quite extensively [13]. But especially these therapy approaches require a very deep understanding of the mechanisms that are active in melanoma cells to get potential targets for the therapy [13]. In this case it not advisable to only determine the genomic profile of the tumor but also the proteomic and metabolomic image. Furthermore, the changes that the whole system undergoes during the disease might have effects on the therapy and therefore are of interest to the current research. Especially when it comes to the late stage of the diseases and cachexia, which is often a great factor that contributes to the death of the patients, the mechanisms are often poorly understood and require much basic research [13].

2.2. Platelets

Platelets are compartments of mammalian blood whose main purpose, to stop bleeding, was first described in the late nineteenth century [3]. Since then the main properties and functions of these cells have been investigated and described. Platelets are nonnucleated cells derived from megakaryocytes in the bone marrow [3]. These cells without nucleus are unique to mammals whereas all other animals' cells related to homeostasis and blood coagulation are nucleated. Unactivated, healthy platelets have a characteristic lens-like shape and are 2-3 µm in diameter [3].

As already mentioned, the main function of platelets is to stop bleeding. An intact endothelium produces certain inhibitors like nitric oxide, which prevent the platelets' activation. But also other pathways are known that ensure the right behaviour of the platelets and form aggregations when needed and in the right amount. When endothelium damage occurs, for example, the platelets come in contact with collagen and adhere to the subendothelium [3]. The binding of the collagen with its receptors on the cells start a signalling cascade, which finalizes in calcium release. This in turn will activate the secretion of the granules, a morphological change and finally the aggregation of the platelets to stop the bleeding [3].

In addition to this coagulative action it is also described that platelets play an important role in the immune system and especially in inflammation. For the reactions occurring during inflammation processes the eicosanoids play a crucial role in all cells [14]. Evidence has been shown that some of the main enzymes important in eicosanoid production, namely COX-1 and COX-2, have an altered expression when platelets are produced during disease. This would also lead to a different eicosanoid pattern secreted during activation [15].

2.3. Platelets and their correlation to diseases

Besides the crucial functions that are executed by platelets to ensure the survival of the organism, platelets have been shown to be involved in many different diseases [3]. On the one hand, platelets show disorders themselves which influence the whole body but on the other hand, they can be linked to other diseases that they are influencing. As the platelets are a compartment of the blood, most of the correlate diseases that are described are blood related or at least originate there [16].

The development of atherosclerosis and the following formation of plaque have been shown to be mediated by blood platelets. The adhesion of the platelets in addition to activation seems to promote atherosclerosis [17,16]. But also peripheral arterial diseases (PAD) show a change in platelet behaviour. In blood derived from patients diagnosed with PAD the platelets seem to be more activated. They show more formed aggregations and higher levels of platelet activation related factors like Thromboxane B_2 [16]. Patients with diabetes mellitus also show a higher abnormality in the behaviour of platelets. The accompanying effects of diabetes like vascular morbidity and mortality therefore seem to be related to the function of platelets as well [18].

Since the function of platelets is also very important in inflammation it is not surprising that also the defence against infections is highly influenced by their activation. But also in chronic inflammatory diseases the role of platelets has been described [3].

2.4. Platelets and cancer

Besides the diseases that are either mediated by a dysfunction of platelets or surely known to be influenced by platelets during their progress there are also other illnesses that can be correlated to them. A malady where the correlation is not so apparent at first sight but was already described in the 19th century is the link to tumor growth and migration [19].

The most important functions that help the tumor cells are the possibility to evade the immune system and to invade the endothelium. Additionally, the platelets have certain pro-angiogenic growth factors that can promote the formation of metastases further. The molecular mechanisms are not fully understood jet but certain animal experiments show that there is a definitive correlation between the formation of metastasis and platelets [20]. Although the platelets are certainly not the only factor that promotes the spread of a tumor it has been shown that mice infected with tumor cells show significantly more tumor growth when the blood platelets are reduced [3]. One crucial part that the tumor cells have to overcome is the evasion of the innate immune system. Natural killer cells (NK) are the major compartment that is capable of destroying circulating tumor cells [21]. Platelets, on the contrary, can evade this mechanism by so called “cloaking” of the tumor cells, which inhibits the destruction through NKs [21,20].

Another crucial part for the development of metastasis is the formation of new blood vessels towards the tumor cells. Among the many different growth factors that are known to promote angiogenesis the vascular endothelial growth factor (VEGF) is one of the most important for

the creation of new blood vessels during tumorogenesis [22]. This growth factor, together with some other signalling molecules, is stored in the granules of platelets. Experiments show that the tumor associated angiogenesis can be reduced when platelet receptors are blocked, thereby inhibiting the VEGF release [22]. In addition to the promotion of angiogenesis, VEGF is also reported to affect vascular permeability, which might lead to an easier invasion of the tissue [22].

2.5. Platelet alteration during diseases

As already mentioned, the role that platelets play during different kinds of diseases is enormous. Especially the negative amplification of tumor cells is a well-studied field and a lot of effort is put into finding the related molecular mechanism. But, on the other hand, the alteration of platelets that might occur during disease has not been studied well so far [6]. Nevertheless, some studies have been published that show a clear correlation between changed platelet expression and diseases. In 2005, a study showed that indeed the release of VGFE in platelets derived from early breast cancer patients is altered [5]. The altered releasate from activated platelets from cancer patients is mostly attributed to an exhausted state of the whole organism. The continuing activation of platelets in cancer patients is a possible explanation for the alteration in released molecules [23]. So far no mechanism that would describe an alteration in the development of platelets has been described.

2.6. Activation of platelets using ionomycin

In order to get access to the eicosanoid pattern of the releasate of the platelets a stimulation of the cells is needed. The natural occurring activation of platelets achieved with the binding of collagen to the receptors causes a signalling cascade that finalizes in a rise of the cytosolic calcium levels. This internal calcium release can also be activated with incubation of the cells with a Ca^{2+}-ionophore like ionomycin, without activating the whole mechanism normally beginning with the receptors-binding [7]. Therefore the activation with ionomycin is a receptor-mechanism independent way to stimulate a fast release of the platelets granules [24]. Since the main interest is not in the secretion of the granules but in the freshly produced eicosanoids, the classical activation of the platelets is only a side effect of the treatment with ionomycin but can be used for the analyses of proteins in the releasate. Nevertheless, the production of eicosanoids is also correlated with the rapid Ca^{2+} release that occurs during

treatment with ionomycin. The pathways that lead to the production of the great variety of eicosanoids is shown in chapter 3.2. In short, the rise in Ca^{2+} concentration activates the cytosolic phospholipase A2 (cPLA2) and therefore releases arachidonic acid from the membrane and makes it accessible to the following enzymes and reactions [25].

3. Lipids and Lipidomics

3.1. Lipid classification and structure

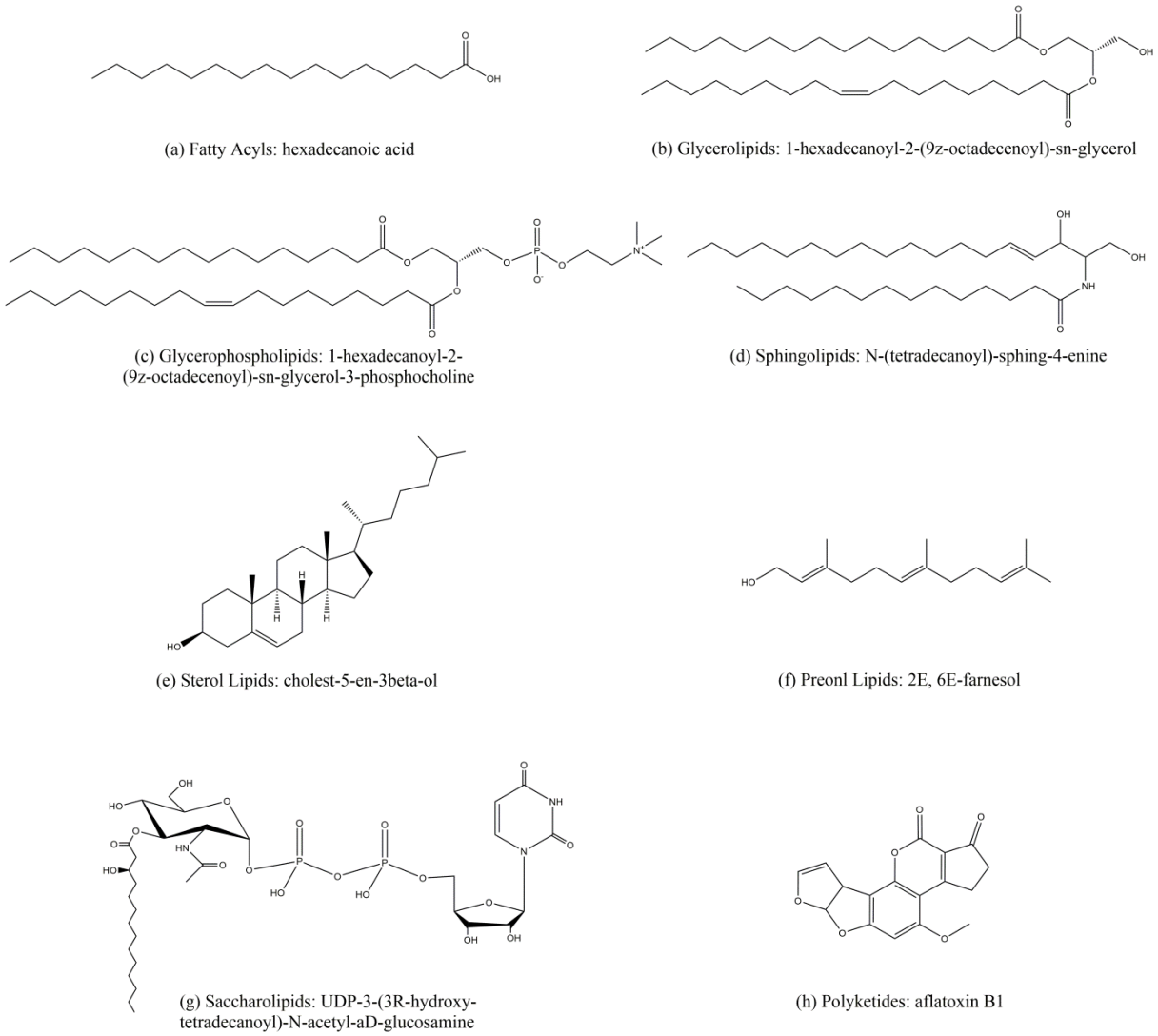

Figure 1: Structure examples for each lipid classes. [26,27]

Lipids can and have been defined in various ways, but the simplest and broadest definition is that they are naturally occurring, mostly water-insoluble molecules that consist of long carbon chains. As it is known today, lipids have an enormous importance in nearly all biological processes. The storage of energy, building of cell membranes or signalling are only a few of

the many functions that are crucial in all domains of life. With the advances in analytical methods and the consequently more precise knowledge, the simple definition of hydrophobic molecules was no longer practical and needed to be updated [26].

A newer and structure-based definition divides lipids into eight major groups: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [26]. In addition to this classification there exists a division into subclasses of the several main groups. These subclasses share a common basic structure but differ in some crucial points like cyclic structures or additions of hetero atoms. The basic structure of this lipid classification is shown in Figure 1.

3.2. Eicosanoids

Eicosanoids are a group of oxygenated, polyunsaturated fatty acids (PUFA) which consist of 18-22 carbon atoms. The main part of this group contains, as the name origin also suggests (greek: eicosa = twenty), twenty carbon atoms and is derived from arachidonic acid. Despite the same origin, this group of lipids has a great variety [26]. These rather different molecules are generated mainly by three enzymes and their pathways cyclooxygenase (COX), lipoxygenase (LOX) and Cytochrome P450 (CYP) but are also created non-enzymatically to a certain extent [25]. The production of eicosanoids starts with the release of a PUFA from phospholipids or diacylglycerol. The Ca^{2+} induced cytosolic phospholipase A2 catalyses this hydrolysis and enables the following stereospecific oxidations mediated by COX, LOX and CYP. Depending on the precursor PUFA and the followed pathway a lot of different eicosanoids can be produced which are divided in different subclasses. Some of the most investigated eicosanoid subclasses, based on structural features are Prostaglandins (PGs), Leukotrienes (LTs), Thromboxanes (TXs), Hydroxy/hydroperoxyeicosatrienoic acids (HHTrE, HETrE), Hydroxy/hydroperoxyeicosatetraenoic acids (HETE), Hydroxy/hydroperoxyeicosapentaenoic acids (HEPE) and Hepoxilins [26,27]. With all their classes, eicosanoids show a variety of biological functions. The processes that are mediated through eicosanoid signalling have been found essential in diseases and inflammation but also in maintaining homeostasis. Thromboxane A_2 (TXA_2), which is a COX1-derived eicosanoid produced by platelets, has been shown to play a role in platelet aggregation and response to injury [25]. Prostaglandin I_2 (PGI_2) catalysed by COX2 enzymes in endothelial cells, on the other hand, shows an inhibition of platelet aggregation [25]. This knowledge is especially

interesting when using a COX1 or COX2 inhibitor, like aspirin for a long term treatment of diseases. Studies show that patients treated with COX1-specific inhibitors show a reduction of blood clotting and patients with COX2-specific inhibitors tend to promote platelet aggregation [25]. These patients are also more prone to suffer from myocardial infarctions or stroke events [28]. This shows that knowledge of the molecular mechanisms related to eicosanoids is a very critical field that is worth investigating.

3.2.1. Prostaglandins

Because prostaglandins show a very short in vivo lifetime of up to 60s they are produced only on activation and are not stored inside the cell. Upon activation AA is released from the membrane and metabolised to PGH_2 by PGHSs [25]. PGHS shows two different enzymatic sites that catalyse the reaction to PGH_2. First it shows a COX activity and forms PGG_2 from AA and two molecules of O_2 and further reduces it as peroxidase to PGH_2 [29]. This again serves as precursor for all the other prostaglandins and thromboxanes whose synthesis is catalysed by different enzymes for every product. The structures and basic pathway leading to a few prostaglandins are displayed in Figure 2. From its production site near the endoplasmic reticulum the PGs diffuse to the cell membrane and are excreted. Prostaglandins are only active in the close surrounding because they get inactivated very fast through oxidation and therefore further marked for the catabolism. They are produced by all types of cells and act in an autocrine and paracrine way to mediate the stimulation of external stimuli like hormones to themselves and surrounding cells [30]. In the extracellular space the PGs bind to G protein-coupled receptors (GPCRs) and mediate their signal to the responding cell.

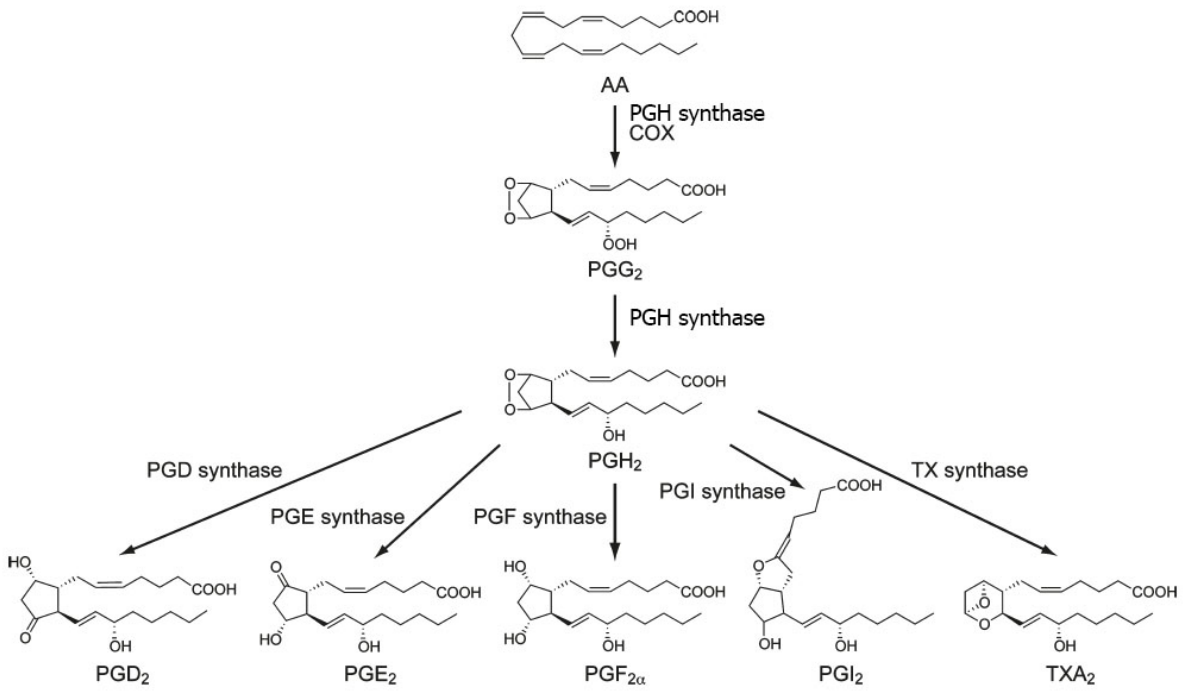

Figure 2: Pathways of prostaglandin biosynthesis. [30]

3.2.2. Leukotrienes

Leukotrienes are also derived from arachidonic acid upon signalling and are not stored in the cell. The first step in the biosynthesis is the insertion of a diatomic oxygen at the 5th carbon atom of AA, catalysed by 5-lipoxygenase (5-LOX) forming the 5-HpETE. 5-LOX also catalyses the dehydration of the same molecule and forms the chemically reactive LTA_4, which is further metabolised to LTB_4 and LTC_4 as shown in Figure 3. Both these leukotrienes are biologically active and are metabolised to other active LTs.

LTB_4 is rapidly processed by cytochrome P450s present in human neutrophils and hepatocytes. The resulting product is 20-hydroxy-LTB_4, which is an agonist for LTB_4 and competes for the binding site of the LTB_4 receptor. Depending on which cell type is present, 20-hydroxy-LTB_4 gets inactivated into different metabolites. The CYP expressed by neutrophils metabolites it further to 20-carboxy-LTB_4 which is biologically inactive.

LTC_4 alternates its biological activity during the metabolism because the initial products, LTD_4 and LTE_4 are highly biologically active molecules that interact with the cysteinyl LT receptors [31].

LTB_4 has been identified as a key player in inflammation due to its effect on leukocytes [32]. When binding to a specific GPCR (BLT1 and BLT2) they alternate the behaviour of their expressing cell. BLT1 for example has been shown to be nearly exclusively expressed on

polymorphonuclear leukocytes [33]. Thereby LTB_4 effects the migration of neutrophils into tissue and the adhesion to vascular endothelial cells. LTC_4 and the metabolic products have been shown to play a role in the contraction of bronchial smooth muscle cells in asthma [32]. They have also been reported to increase the vascular leakage, which further on often leads to edema [32,33].

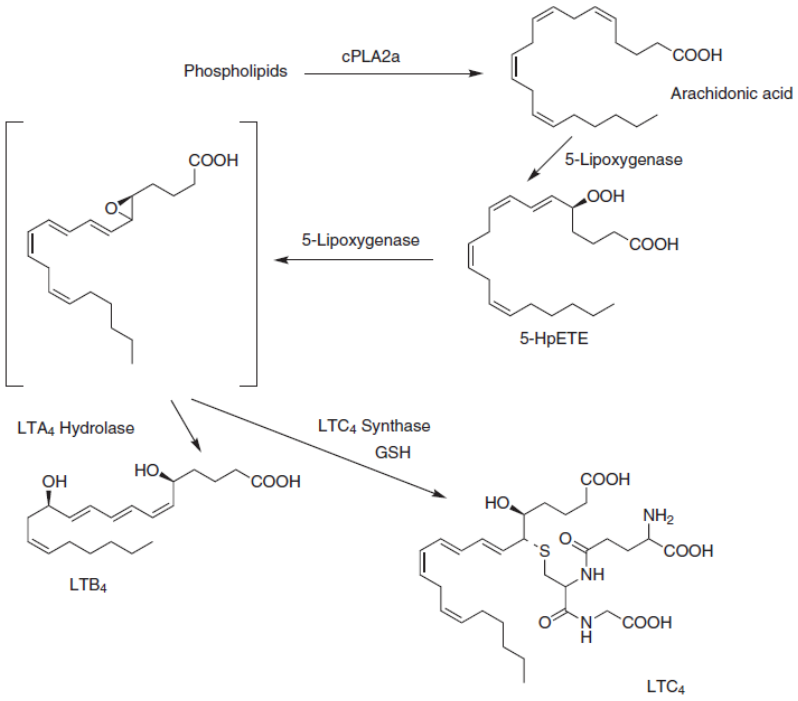

Figure 3: Pathway for the biosynthesis of leukotrienes from arachidonic acid. [25]

3.2.3. Hydroxyeicosatetraenoic acids (HETEs)

The most important formations of HETEs are catalysed by lipoxygenases with the intermediate HpETEs. The major sites of oxidation for AA are at the 5, 12 and 15 position and are catalysed by 5-LOX, 12-LOX and 15-LOX respectively [25]. The majority of the products are synthesised in S configuration although there are a few with other conformation with biologically distinct functions from their S isoforms.

The 12S-LOX catalyses a pathway that is normally only found in platelets, keratinocytes and specific tumors [34]. This stereospecific synthesises 12S-HpETE from AA and further to 12S-HETE. The 12R-LOX which is only found in human skin normally catalyses the formation of ω-hydroxyceramide that is essential in the composition of mammalian skin barrier. Anyhow it is believed to also create the 12R-HETE, which can be found in psoriasis where it may play a role for the disease [34].

The 15S-HETE in humans is formed mainly by 15-LOX-1 and 15-LOX-2 but can also be formed by other enzymes to a certain extend. COX-1 and COX-2 are reported to create small amounts of 11- and 15-HpETE which are subsequently oxidised to their HETE products [35]. The reactions again show a certain stereospecificity in regard of forming the S- or R-enantiomer. But whereas 11-HETE is catalysed exclusively in the R-form by both COX-1 and COX-2, 15S-HETE is created by COX-1 and COX-2, and 15R-HETE by COX-2 enzymes only. Interestingly, the formation of 15R-HETE is not blocked by aspirin although the formation of PGs by COX-2 is efficiently disabled, which then becomes the major AA metabolite [35].

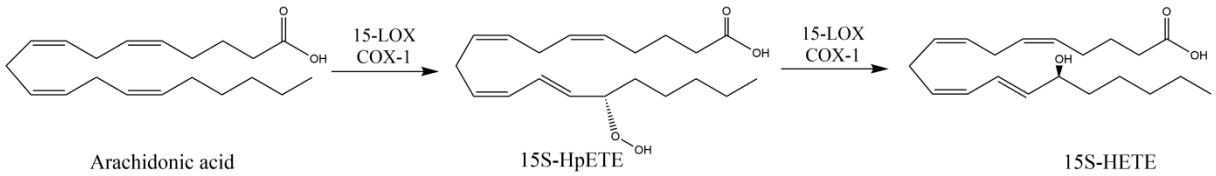

Figure 4: Enzymatic formation of 15S-HETE as example for the production of HETEs.

3.2.4. Eicosanoids in diseases

Eicosanoids have been described as signalling molecules in many biological processes. Especially in disease their role has been investigated intensively in the last few years [25]. The change in lipid signalling and its effects during inflammation is a well-described field within eicosanoid research [28].

Many of the pro- and anti-inflammatory impacts of the lipids, derived from the COX pathways are correlated to the most observed signs of inflammation like pain or swelling [36]. But also the LOX enzymes are reported to be involved in specific aspects of inflammation. Important mediators for this inflammation-related signalling are prostaglandins, leukotrienes and thromboxanes [37]. These are described to also promote different diseases that are directly correlated to inflammation but also cancer, obesity or cardiovascular diseases [38].

Studies and experiments on model organisms show that there is a strong correlation between inflammation induced through certain eicosanoids and cancer. PGE_2 levels in the serum for example show a significant change in patients with certain types of cancer before and after treatment [39]. Also the treatment with specific COX-2 inhibitors shows a change in the progression of tumors and improves the survival in some patients. But also other drugs have been developed that either promote the degradation of PGs or inhibit their receptors and therefore impair tumor progression. Besides the well-studied COX eicosanoids also

metabolites derived by LOX and CYP enzymes have been shown to have an effect in cancer diseases. Within the many different LOX enzymes that are known, some of them like 5-HETE, 12-HETE and 20-HETE have been reported to show pro-cancerogenic properties [40]. 8-HETE and 11-HETE, on the other hand, show tumor suppressive activities within certain types of cancer [41]. To fully understand the processes that lead to pro- or anti-tumorogenic effects further investigations have to be made. Nevertheless, the knowledge that the targeting of inflammation processes might change the behaviour of tumors gives rise to new potential drug targets. Many of the inflammation pathways are indeed already targets when treating chronic inflammations or cardiovascular diseases.

3.3. Lipidomics

The research of lipids has proven to be one of the most challenging fields. Compared to other main structures that are crucial to the life we know today, like nucleotides and proteins, the high-throughput analysis of lipids has the shortest history. In the last decades the analytics of biological functions and structures has evolved very strongly for all omics fields. The complexity of the omics fields and therefore the problems arising for the analytical approaches is somehow similar to the biological dogma, DNA→RNA→Proteins→Lipids, and will be explained in this order.

The first approach to understand the complexity of life was to understand the meaning of the DNA, as the molecule that is given from a parental cell to the progeny. With advances like PCR and labelled nucleotides the sequencing of DNA has become a routine procedure today. With the newest approaches it is possible to get the sequence of a human DNA within hours to days starting from one single cell. Furthermore, the process is quite cost-efficient [42].

When moving forward to RNA a new big problem occurs to the analytics, which is the higher dynamic range. While every healthy cell provides exactly one copy of DNA, the concentrations of RNA can vary quite a bit, which makes an overall view of the transcriptome very difficult.

But not every RNA molecule has to be translated into a corresponding protein and so the analytics of protein is inevitable in order to provide a more exact picture of a cell's life. In this field the analytic has to face an even higher dynamic range and additionally has to deal with the certain heterogeneity. The chemical properties of DNA and RNA are only defined by 5 different building blocks whereas proteins consist of more than 20 different amino acids which provide different chemical structures. Furthermore, biologically active proteins tend

towards modifications that might seem negligible but in fact often make the difference between an active and an inactive molecule. Hence, proteins can have very different properties concerning hydrophobicity or structure. But with the advances in chromatography and mass spectrometry it is possible to analyse a large amount of proteins in a certain cell. Nevertheless, certain protein families, like for example membrane proteins, can be an analytical challenge and still need research and enhancements.

The high-throughput analysis of lipids is the latest addition to the omics of the biologically relevant macromolecules. As in proteomics the dynamic range is quite high, which makes it problematic to analyse very low abundant lipids. But especially for signalling molecules even very small amounts can trigger pathways and are therefore worth investigating. Another important issue is hydrophobicity of the molecules. Unlike all the other mentioned biological structures lipids are often not or very bad water soluble and therefore need to be dissolved in organic solvents. This can provide problems with the sample preparation protocols. Many of the used consumables likes tubes or tips are designed to be water repelling, and therefore attract hydrophobic molecules. On one hand this can lead to a loss of many lipids during the sample preparation for they are sticking to the sample tubes. On the other hand some organic solvents that are good for the extraction can dissolve the storage tubes and consequently contaminate the sample [8]. But the problem is that some of the lipids are not only hydrophobic but also consist of a hydrophilic, charged part. This dualism makes it hard to find an appropriate solvent. Some of the lipids also tend to form aggregations which make the analysis with HPLC-MS further difficult.

Another difficulty that is to overcome is the similarity of the lipids. When speaking of eicosanoids, which is the main interest in this work, almost all of the hundreds of existing lipids origin from the same few molecules, which is arachidonic acid or other ω-3 and ω-6 polyunsaturated fatty acids. This leads to many isobaric eicosanoids, which are naturally difficult to detect via mass spectrometry. In addition, the metabolites show a very high resemblance concerning their structure. An oxidation, for example, can occur on many different parts of the molecule. The differences in the final eicosanoids can be as insignificant as the position or the stereochemistry of a hydroxyl group, but nevertheless can have very different biological activities and therefore need to be distinguished.

For the signalling molecules, it is also in their nature to sometimes have a very short lifetime. That’s why the samples need to be handled with a certain caution so as not to lose information or create any artefacts [43].

3.4. HPLC-MS/MS in the perspective of lipidomics

High accuracy mass spectrometry has been proven to be a very powerful tool for bioanalytics. Hence it's hardly surprising that also the field of lipidomics has been developed using MS. Mass spectrometry offers a variety of possibilities that are used for different approaches all over the analytical field. Starting with different ionisation sources over several fragmentation and separation methods to a number of various detectors, many of them were used in lipidomics and tested for their capability in this discipline. The technique that is mostly used today, and was also used in this work, is high performance liquid chromatography coupled with mass spectrometry [8].

3.4.1. Sample preparation

Sample preparation, starting with collection of the sample through storage to extraction of the lipids and some cleaning steps, is considered the first crucial step that ensures a good lipidomics analysis. Depending on which method of analysis is used, sample preparation has to be different, especially concerning the amount of impurities that are in the sample. Samples prepared for shotgun lipidomics have to be significantly cleaner compared to samples that are analysed via HPLC-MS/MS because every additional impurity influences the ionisation efficiency.

Sampling

When starting with an analysis, the first step has to be, of course, the sampling. While the sampling of cell culture samples is rather easy and does not contain many sources for major mistakes, the sampling of compartments from biological systems can be quite tricky. When analysing a human organ, for example, sample taking should be done very carefully to ensure a representative analysis. An organ does not consist of one specific cell type that is isolated from all other compartments but is a network of different cells that are organ specific and unspecific. Compartments like conjunctive tissue or endothelial cells are to be found in nearly all parts of the system. Moreover, blood as a carrier of many different lipids is one of the major contamination sources that might falsify the analysis. Additionally the size of the sample should be big enough to ensure an analysis of high quality but also small enough to minimize the damage of the organ. The problem again is the heterogeneity of the biological

compartments. A sample taken from unspecific cell compartments will sophisticate the whole analysis [8].

Storage and handling

As with all biological samples, storage is a critical point of sample preparation that can falsify the results. The best thing to do is to not store the samples but analyse them immediately, which is of course not always possible. Especially for signalling molecules like eicosanoids there exist a lot of enzymes that degrade the lipids and therefore will change the amount or species of lipids. The best method to overcome these effects that is widely used for all kinds of biological samples is to store them at low temperatures. For short time storages a temperature of -20 °C is reasonable but does not stop all degradation processes and lower temperatures are sometimes recommended. For longer storages a good way to overcome many of the enzymatically caused degradations is to deplete the proteins and extract your lipids of interest.

But not only the degradation of the lipids can be problematic when storing them for longer times. Also the molecular oxygen induced auto-oxidation will alter the lipid composition of the samples. Especially unsaturated double bounds are vulnerable to these kinds of oxidations. For the analysis of eicosanoids this is a very big problem, because the distinct molecules sometimes differ only in the number or position of hydroxyl groups. Therefore, this change has to be minimized in order to ensure a representative analysis. A good way is to work and store the samples in a nitrogen atmosphere or add some antioxidants to the liquid.

A further problem that can occur during storage of lipid samples is the decomposition of the storing containers. For some lipids it is necessary to solve them in organic liquids, which are sometimes not compatible with the regular used consumables and tend to dissolve them during long term storage. Although this is an inconvenience because it varies from the standard protocols that are used in bioanalytics it can easily be overcome by using glass containers for storage or glass ware for handling the samples [8].

Lipid extraction

To get access to the lipids and prepare them for an analysis via mass spectrometry it is inevitable to separate the interested species from other biological compartments and inorganic salts. Depending on what sort of biological sample has to be analysed and which is the lipid

class one is interested in, many different extraction methods have been used. Since the possibilities of combinations are numerous, for a new analysis the best solvent combination has to be evaluated. The critical point for the selection of the extraction method is, on the one hand, the properties of the lipid. As shown in Figure 1 the different lipid classes have many different properties which can help separate them form the others. A lot of the lipids show a structure where they have a head group with higher polarity and a side chain with lower polarity. This can be used for the selection of the combination of the solvents. Hence, polar solvents are chosen for lipids with higher polarity and nonpolar solvents for nonpolar lipids to maximise the yield. The second property that the extracting solvent has to provide is the possibility to dissolve the cellular compartments. To get access to the lipids inside a cell or even inside the cellular compartments of a cell, the walls have to be permeabilised or destroyed. Furthermore, some lipids tend to get trapped on some cellular compartments and stick to them through unspecific bindings. Sometimes the extracting solvent is not proper for the freeing of all lipids and the matrix of the cell has to be destroyed otherwise. This can either be done by heating the samples or mechanically destroying the compartments using for example ultrasound. Although this will definitely achieve the dissolution, it has to be used with caution so as not to destroy any lipids of interest or create artefacts [8].

Liquid-Liquid extraction and solid phase extraction

The samples that are obtained through lipid extraction are not suitable for analysis via HPLC-MS/MS. For this purpose an additional cleaning is necessary prior to the analysis. The liquid-liquid extraction and solid phase extraction (SPE) have been proven to be very potent. Here again the main criterion for the choice of the method is which lipid species has to be examined. The reasons why a sample should be pre-cleaned are numerous but the most important is to get rid of the many additional impurities that occur during extraction. Especially for the direct infusion of shotgun lipidomics but also for HPLC methods inorganic salts can have a great influence on the analysis. Particularly the ionisation efficiency is affected by high salt concentrations. But also other biological compartments like proteins or nucleotides, as far as they have not been depleted during the extraction, should be removed in order to create a clean sample. It is also possible to furthermore select between different lipid classes and species when a certain hydrophobicity is chosen.

For the analysis of eicosanoids the SPE has been proven to be a very powerful tool. An often used method uses reversed-phase columns with C18-materials. These methods show a very

high recovery when using the right solvents for the washing and eluting steps while removing most of the unwanted impurities [43,8].

Internal and external standard

When aiming to quantify either absolutely or relatively, the preparations to do so have to be done right at the beginning of the sample preparation. Absolute quantification of lipids derived from the sample is naturally a very challenging task. In addition to the creation of concentration curves derived from lipid standards also the ionisation efficiency in the biological matrix and the recovery of the lipids during sample preparation have to be known to certain extent to allow for representative quantification. Nevertheless, an exact quantification of a lipid from a biological source is possible yet inconvenient.
When a quantitative result has to be obtained it is inevitable to use a quality control to ensure the correctness of every step from sample preparation to the analysis via mass spectrometer. The best way to do this is the usage of one or more internal standards. The properties of these standards should be chemically and physically as close to the analytes as possible. This ensures that all the steps like extraction, separation and ionisation have a certain quality control and changes within these are recognised and can be considered for the quantification. The best option for a chemically and physically similar behaviour while a difference in mass spectrometer is visible is an isotope labelled standard. The isotopes that are mostly used as internal standards are either C^{13} marked or deuterated (H^2). When analysing species that are quite different it is advisable to use at least one standard for each class of lipids. This will ensure that all analytes are controlled during the processing [8].

3.4.2. Shotgun lipidomics

The groups of Han and Gross [44] and Ejsing [8] first used the term shotgun lipidomics for their platforms based on direct infusion and therefore characterised the name. The simplest form of direct infusion into a mass spectrometer is with a syringe pump. Although these systems provide a constant flow over time they bring some drawbacks with them. A major disadvantage of this infusion method is the relatively high flow rate that has to be assured. This requires a high amount of sample that cannot always be achieved especially for biological studies. Furthermore, the bad compatibility with automation and the easy clogging

of the capillaries make this method inappropriate or at least inconvenient for high-throughput analyses.

Newer, chip-based devices can evade many of these drawbacks and therefore make a direct infusion based high-throughput analyses for lipidomics possible. These chips consist of several hundred little electrospray nozzles with constant diameter embedded on a plate. One major advantage is the very low flow rate of down to ~100 nL/min that can be achieved [8]. Furthermore, automation is much easier and the risk of clogging and cross-contamination is minimized. The drawback of this method is the high cost of these chips.

The aim for shotgun lipidomics is the high-throughput analyses of lipids derived from biological samples using the distinct properties of the lipid classes and species. The exact quantification of specific lipids or lipid classes in a biological matrix is one of the major advantages that can be achieved with a direct infusion.

3.4.3. High performance liquid chromatography

Although shotgun lipidomics has its advantage in speed and constant flow over long time, for biological samples it is sometimes necessary to reduce the complexity so as to not miss out on some important metabolites. For this reason, the introduction of HPLC as a separation method prior to the MS is the logical consequence, since it is a standard procedure for proteomic research with a massive amount of analytes.

This not only provides separation according to the chemical properties but also gives another tool for an accurate identification of a certain lipid in a complex biological matrix. Nearly all common column types including normal-phase, reversed-phase (RP), ion-exchange, hydrophilic interaction, etc., have been used in order to achieve separation of the lipids [45]. As with other biological molecules, normal-phase and reversed-phase have proven to be very useful and are widely used today. While normal-phase is mostly used for separating different lipid classes, reversed-phase chromatography can be used to separate certain lipid species within the classes [46,47].

For the separation of the eicosanoids in this work reversed-phase chromatography was used. The RP column provides a non-polar stationary phase and a relatively polar mobile phase. This chromatographic specification makes possible a separation of the lipid molecules based mainly on their hydrophobicity and less on their polar head groups. Therefore, the length of the fatty acid chain, the numbers, positions and configurations of the double bounds and additional heteroatoms of the chain determine the separation. Since the fatty acid chains and

the small differences within the different lipid classes dominate the interaction between the molecules and the stationary phase, this method is suitable for the analysis of eicosanoids [47].

3.4.4. Ionisation methods

Matrix-Assisted Laser Desportion/Ionisation (MALDI)

Matrix-Assisted Laser Desorption/Ionisation (MALDI) is a soft ionisation method that has been used for lipidomics research since the late 1980s [8]. In the following years of research the method was used in positive and negative ion mode and it showed great advantages for several lipid species. Spotting lipid samples is relatively easy and a result can be obtained in a very short time. It also shows very high sensitivity, based on good ionisation efficiency, although in complex biological matrices some lipid classes show better ionisation and therefore quench the ionisation for others. Also, ionisation of the matrix and the following masses in the low m/z range are a drawback for the method. The occurring post source decay is also used on the one hand but can give unwished side-effects on the other hand. The main reason why this method is not used for the analysis in this work is simply the bad compatibility with the HPLC system that is used before the mass analyser.

Electron Spray Ionisation (ESI)

For both direct injected shotgun lipidomics and HPLC coupled lipidomics, Electron Spray Ionisation (ESI) is a suitable source for ionisation.

The analytes solved in a liquid are introduced into the ion source via a capillary. High voltage that charges the liquid drops that are formed when releasing the liquid into the vacuum is applied to the tip of the capillary. Through the high charge and the vacuum the drops subsequently ‘explode’ to form smaller drops. This happens until all the liquid has evaporated and the analytes remain with a multiple charge [48]. Depending on the applied voltage the source can be run in positive or negative ion mode. In positive ion mode the capillary tip carries the positive pole and the entrance to the mass analyser the negative pole. The positively charged analytes are therefore driven to the negative plate in the direction of the analyser. A schematic picture of an ESI source in positive ion mode is depicted in Figure 5. For negative ion mode the settings are reversed, namely the capillary carries the negative

pole, the plate in the direction of the analyser the positive pole and the analytes are negatively charged.

In addition to the good compatibility of the source to the HPLC methods, ESI provides a few advantages that make it very interesting for lipid analysis. The source is one of the least destructive ionisation methods and hence shows very little in-source fragmentation. The mostly intact lipids that leave the source show only the expected masses and allow for good identification and quantification. With this high amount of structurally undamaged metabolites, the fragmentation source determines the degree of fragmentation.

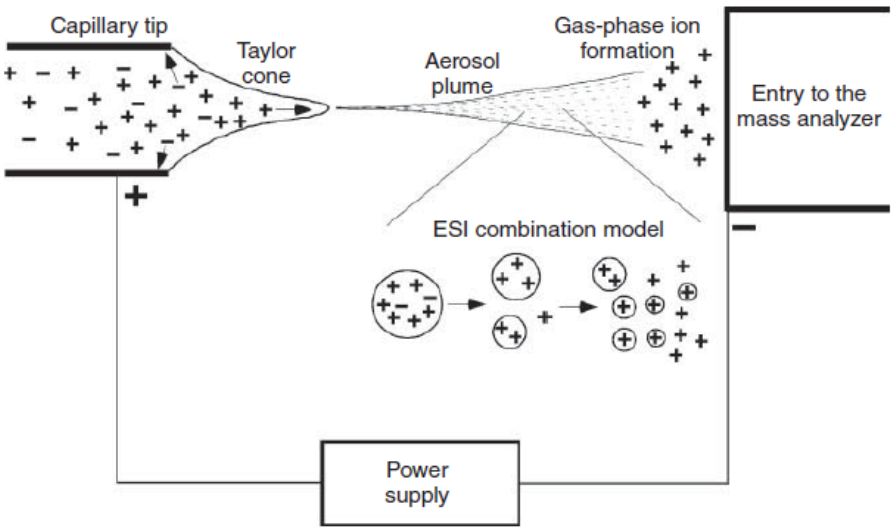

Figure 5: Schematic picture of an ESI source used in positive ion mode. [8]

3.4.5. Mass analyser

The possibility for mass analysers that separate ions based on their mass to charge ratio (m/z) is enormous. All of the analysers with their advantages and drawbacks have been used in the history of lipidomics. Some of the most important properties that are essential for lipidomics research are high mass accuracy and resolution [9]. The Time-of-flight (TOF), Quadrupol and Ion Trap are some of the most common analysers that are used today, but are often used in combined devices.

As depicted in Figure 6, the Q Exective Orbitrap is a mass analyser combined from several modules. As an ionisation source an ESI is used that leads the ionised analytes into the RF lens where they are focused and leads the ions further to the first Quadropol analyser. The Quadropol is designed to stabilise ions of a certain m/z ration through a radio frequency. This can either be used to stabilize the path of all ions or to select ions with a certain m/z ration and

send them further to the C-Trap. As an ion trap, a C-trap is able to collect the ions and release them again selected by m/z ration into the next modules which are either the Orbitrap analyser or the collision cell. In the collision cell the injected ions are brought to collision with a collision gas at a certain energy to induce fragmentation and send fragments back to the C-trap and further to the Orbitrap. The Orbitrap in this system serves as the mass analyser that either analyses the ions from the ion sources directly or the fragments after the CID. Therefore, the ions are brought to a circulation movement around the core part of the Orbitrap. The frequency of the circulation is then transformed via Fourier transformation (FT) into a m/z ratio for all the ions present in the analyser. This setup allows for many different settings in selecting ions and therefore is widely used for bioanalysis.

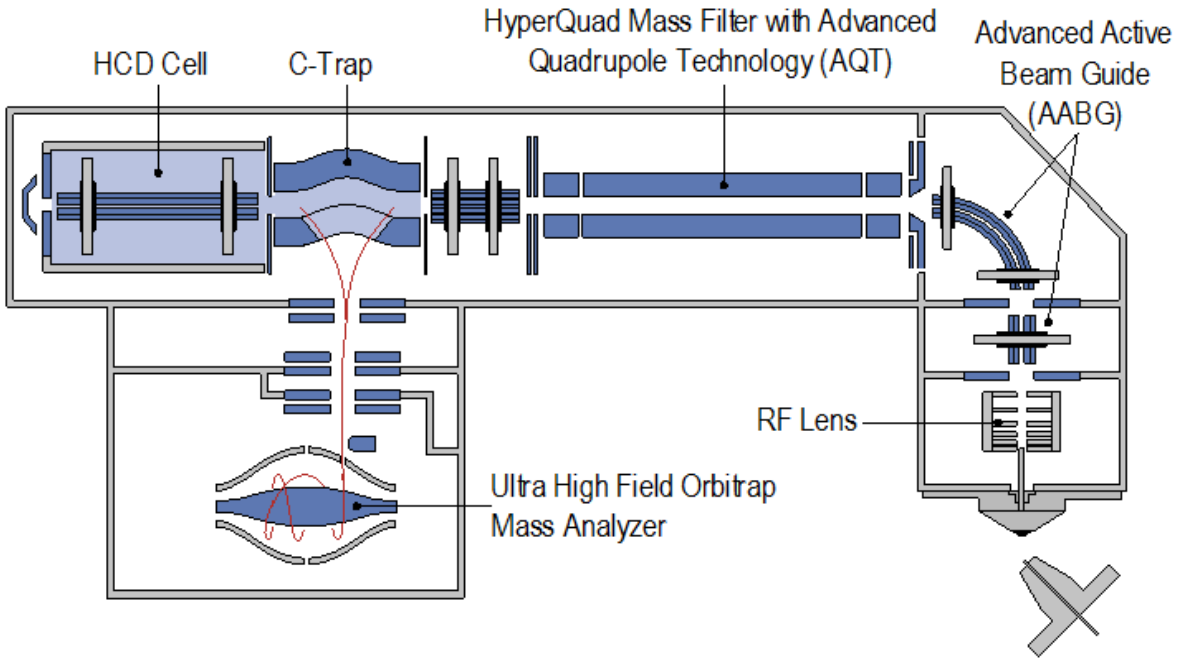

Figure 6: Picture of the function of a Q Exective Orbitrap.[scheme from thermofisher.com]

Depending on which analyser selects a specific ion, four different settings are possible to analyse a sample, namely Product ion, Neutral-loss scan (NLS), Precursor-ion scan (PIS) and selected reaction monitoring (SRM). These settings are schematically shown in figure 7.

(a) Product-ion analysis

For product-ion analysis one precursor ion is chosen and sent to the collision cell. This setting is used for the analysis of a special analyte within a mixture. The chosen ion can be collected

in the C-trap until enough ions are present to ensure good fragmentation. This method allows the creation of a fragmentation pattern and correlates a precursor mass of the sample with a fragmentation pattern. This can help to relate the sample to known MS/MS spectra or give novel structural information of the chosen precursor ion. Furthermore, this can be used in an iterative way, namely if a certain fragment is chosen again and sent to the collision cell [8].

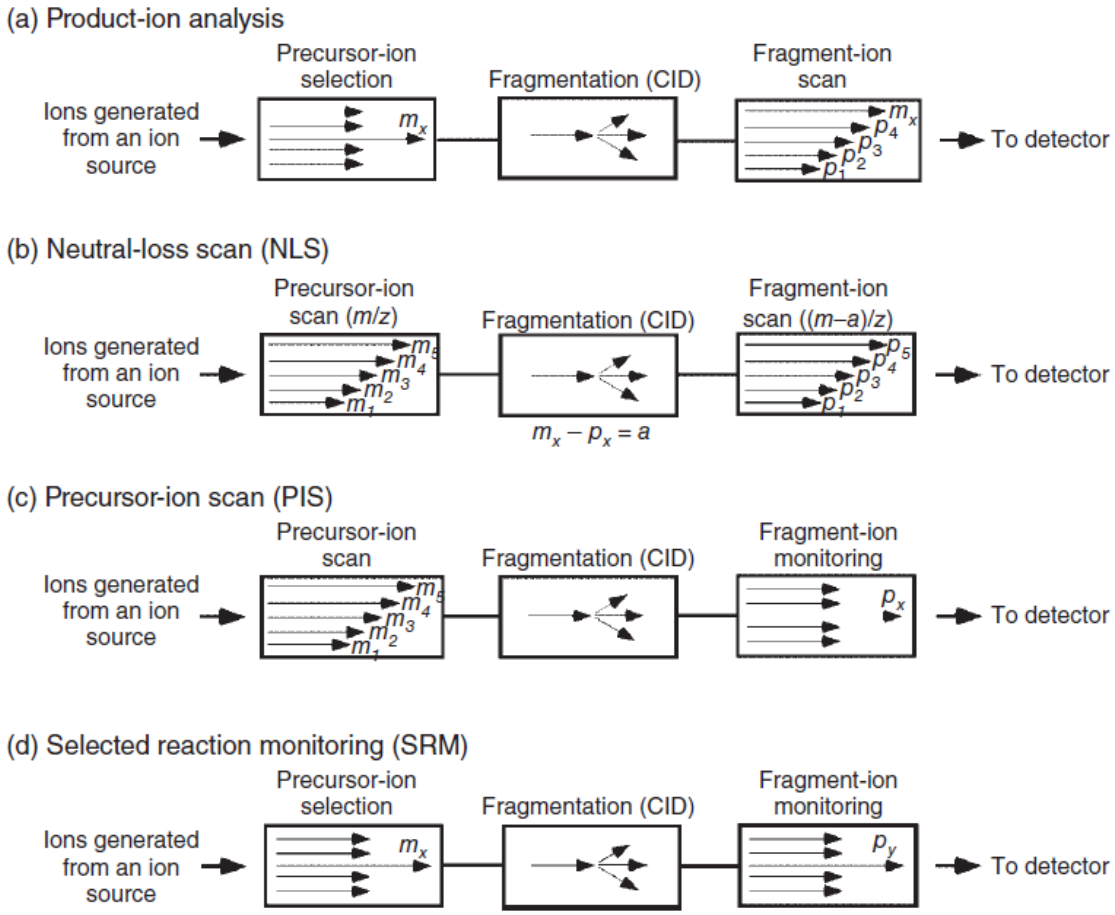

Figure 7: Schematic of possibilities for ion selection settings [8]. The shown tandem mass spectrometry techniques are shown as they are used in an QqQ-system, but the principle can be used for all tandem mass spectrometry system with two analysers and a collision cell. 'm_x' stands for the ions of the precursor, 'p_x' is the ion after the fragmentation, and 'a' is the neutral loss.

(b) Neutral-loss scan (NLS)

For the neutral-loss scan both analysers are scanning simultaneously but do not detect all the ions. The first analyser transmits one precursor mass after the other into the collision cell. The second analyser is set to only detect a mass that shows a specific offset to the precursor. The mass that is lost, the so-called neutral loss, does not carry a charge and is therefore lost during the fragmentation. This absence of a specific mass 'a' is used in lipid analysis to scan for

specific lipid classes. Some classes tend to lose a certain part of their structure which is often within the head group of the lipid class [8].

(c) Precursor-ion scan (PIS)

The precursor-ion scan mode works similar to the NLS method. The difference is that the second analyser does not scan for the neutral loss, but for a special fragment that is expected. Analogous to the NLS mode the PIS is good for use in shotgun lipidomics to scan for known similarities and therefore dedicates the analytes to certain lipid classes [8].

(d) Selected reaction monitoring (SRM)

For selected reaction monitoring both analysers only detect one specific ion. The first analyser is set to only transmit one special precursor mass into the collision cell. The second analyser detects only one particular ion as well. This is used to achieve high specificity and sensitivity. When setting the first, the second or both analysers to detecting multiple ions the setting is referred to as multiple reaction monitoring (MRM), which is a widely used technique today [8].

3.4.6. Analysis of acquired data and bioinformatics

While the acquisition of qualitatively high data is the most important part during the analysis and has to be enhanced steadily, evaluation of the data is a crucial point as well. Since the field of lipidomics is rather new compared to proteomics research, automated data evaluation is not as advanced.

The basis for an automated identification of lipids is a database that lists the known analytes and makes an assignment to created MS data possible. To ensure the correct identification of an analyte the libraries contain the exact mass, isotope patterns, MS/MS fragmentation patterns and if coupled with LC the retention time. A database containing over 40000 unique lipid structures is the LIPID MAPS Structure database (LMSD) which is publicly available at www.lipidmaps.org/data/structure. Although this is a good starting point for the analysis the database lacks experimental data for some of the lipids, which makes identification difficult. In addition to the libraries and databases there is also the need for software to ease the following data processing and make high throughput possible [8].

3.4.7. MS/MS characterisation of eicosanoids

The exact identification of lipids from mass spectrometry is very challenging due to its tendency toward isobaricity. Since a lot of eicosanoids are derived from the same PUFA the differences are often only in the position of a hydroxyl group or a double bond. The HETEs for examples show 13 bioactive isoforms that only differ in the position of their hydroxyl group on the side chain [35]. To identify these eicosanoids all together the separation via HPLC has to be very good since the hydroxyl groups are pretty close together and the difference in the behaviour in the LC are sometimes minimal. But in addition to this, fragmentation through collision induced dissociation can give the needed information to ensure identification. But compared to the fragmentation of other biological molecules the fragmentation of eicosanoids is not as generalisable. Proteins for example show a very distinct fragmentation pattern. This makes it even possible to identify proteins that are not in the database and discover their sequence de novo. Also for other lipids and metabolites it is possible to find structures that are common in the fragmentation pattern and to make identification easier. Non-esterified fatty acids for example show some common peaks for the CID and also tend to show specific patterns related to the position of the double bond [49,50]. Nevertheless, it is possible to detect differences in the fragmentation patterns of eicosanoids and to distinguish different isomers with it. But this assignment cannot be done de novo but has to rely on a database where the dissociation of the eicosanoid has been saved with the same or related conditions.

3.4.8. Applications for health and disease

Lipidomics as an analytical tool can be used for basic research as well as for applications in clinical research. Since the changes that occur during diseases are correlated to changes in the lipid metabolism lipidomics research can give an overall insight into a diseased system. The fields where this kind of global analytics is utilised can be quite versatile. Lipidomics can give an insight into the change of the lipid metabolism that occurs during treatment with a certain drug. Also the overall change of the system of the alteration of an organ specific pathway that arises during the development of a disease can be measured. Furthermore, with its abilities to discover unexpected metabolites or patterns it is also used to find new biomarkers [8]

4. Materials and Methods

4.1. Purification of blood platelets and activation with Ionomycin

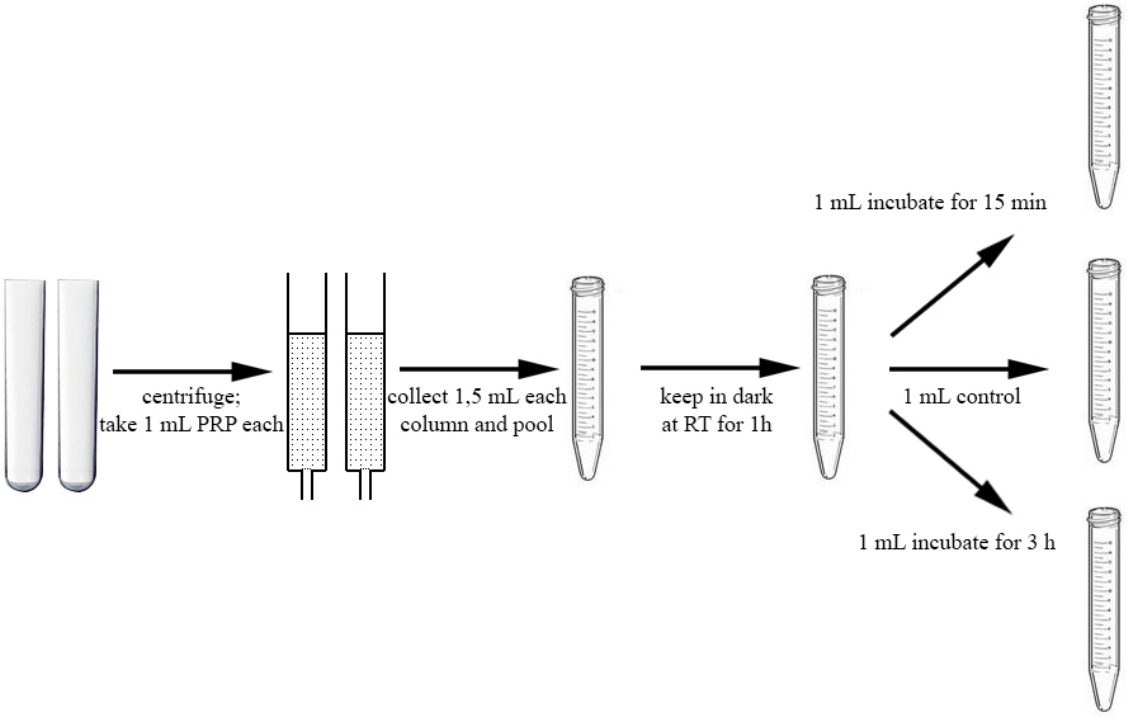

Figure 8: Schematic workflow of the platelet purification and activation.

For the purification of the platelets peripheral venous blood was collected into two tubes containing 3,2% sodium citrate (Vacuette system; Greiner, Kremsmuenster, Austria). To get non-activated platelets, the blood was collected into pre-opened tubes, letting it drop freely after the first few drops were discarded. After closing the tubes, they were gently inverted four times in order to ensure a mixing with the sodium citrate without activating the platelets. Afterwards the tubes were centrifuged at 100 g for 20 minutes to achieve platelet rich plasma (PRP). About 1 ml of PRP per tube was used to purify the platelets using a size exclusion chromatography. Therefore 10 ml of sepharose 2B (Sigma, Germany) were mixed with 10 ml of FCS free RPMI medium (RPMI medium 1640 (1X) [+]-Glutamin, Gibco, UK) and filled into plastic columns. 1 ml of PRP was loaded onto the prepared column and eluted with RPMI medium. After discarding the first 2,5 ml, a fraction of 1,5 ml was collected, pooled with the second tube's fraction of the same donor and kept in a dark place. After one hour the platelets suspension was divided into three fractions. One fraction was used as a control and the other two were activated with 100 µl of 10 µM Ionomycin (Sigma, Germany) dissolved in PBS and incubated for 15 minutes and 3 hours at room temperature.

4.2. Sample preparation with solid-phase extraction

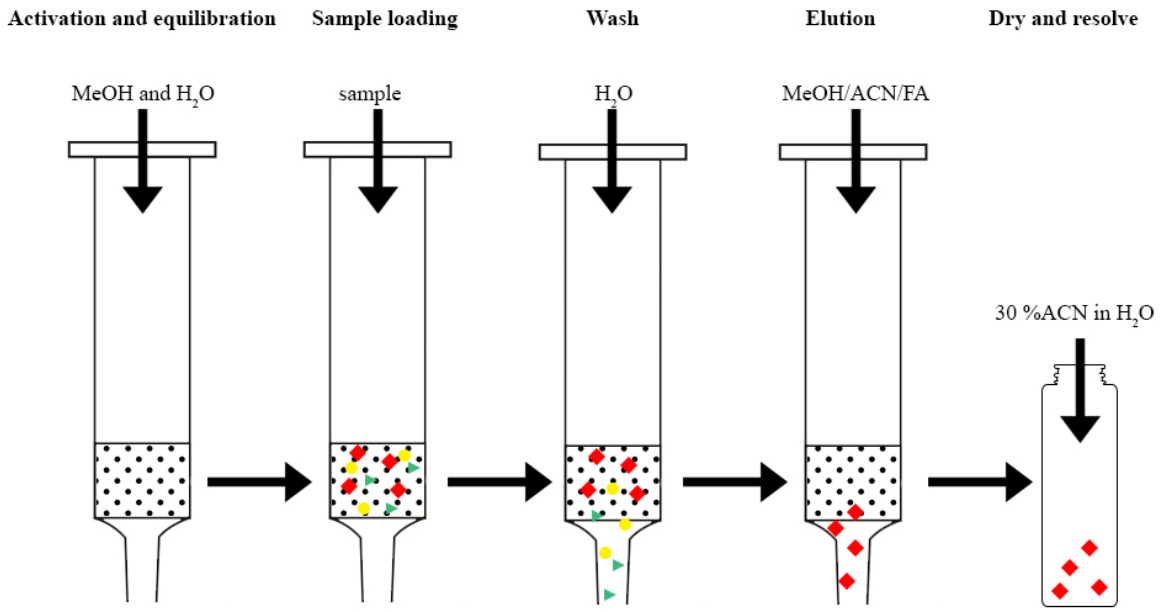

Figure 9: Schematic workflow of the solid-phase extraction.

After the incubation time the samples were centrifuged at 1500 g for 10 min to separate the platelets from the secretome. The supernatant was decanted into a new 15 ml tube and 4 ml of ice cold absolute ethanol was added to it and incubated at -20 °C overnight in order to precipitate the proteins. After at least 18 hours the samples were centrifuged at 4750 g and 4 °C for 20 minutes and the supernatant filled into a new vial. The protein pellets as well as the platelet pellets were dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4% CHAPS, 0.05% SDS, 100 mM dithiothreitol). Additionally the platelets were lysed with sonication using an ultrasonic homogenizer and used for proteomic analysis, which is not shown in this work. The ethanol of the supernatant fraction was evaporated in the SpeedVac at 37 °C and the sample volume reduced to about 1ml. The remaining liquid was diluted with cold water to 3 ml to ensure an ethanol amount of fewer than 15%, which is necessary to ensure a good SPE. 5 µl of internal standard lipids (Table 1) were added. To extract the eicosanoids a solid-phase extraction was performed using a C18-column. Therefore the columns were activated with 2 ml Methanol and equilibrated with 2 ml H_2O. After that the samples were loaded onto the column and washed twice with MS-grade water. Finally the lipids were eluted with 2x250 µL elution solution (49,5% MeOH, 49,5% ACN, 1% FA). For the whole extraction process the column never dried and the liquid went through at about 1 drop per 2 seconds. To enable an exact quantification of the lipids, the eluted sample was dried completely in the SpeedVac at 37 °C and resolved in exact 200 µL 30% ACN in H_2O + 1% FA.

Standard lipid	MW	MW in neg. ion mode	Concentration [µmol/L]
5S-HETE-d8	328.2845	327,2781	76,2
PGF2α-d4	358.2653	357,2585	139,6
PGE2-d4	356.2497	355,2428	140,4

Table 1: Deuterated eicosanoids as used for the internal standard

4.3. Analysis of the samples using HPLC-MS/MS

4.3.1. Materials

Vanquish UHPLC System (Thermo Fisher Scientific)
Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific)
Kinetex® 2.6 µm C18 100 Å, LC Column 150 x 2.1 mm (Phenomenex)
Eluent A: H_2O + 0,02% FA
Eluent B: 10% MeOH + 90% ACN + 0,02% FA

4.3.2. Liquid chromatography method

For the liquid chromatography a Vanquish UHPLC System (Thermo Fisher Scientific) was used with a Kinetex C18 column (Kinetex® 2.6 µm C18 100 Å, LC Column 150 x 2.1 mm (Phenomenex). The used method was 45 minutes long and used a gradient of eluent A (H_2O + 0,02% FA) and eluent B (10% MeOH + 90% ACN + 0,02% FA). The samples were analysed directly after the preparation with the SPE and were not frozen in between. Since the method is pretty long the samples were stored in the cooled (4 °C) auto sampler of the LC system. For the analysis 5 µL of samples were loaded onto the column and eluted with the gradient displayed in figure 10 and table 2 at a flow rate of 0,5 mL/min. All the samples were injected twice and before a new sample was injected a whole method was run with a blank sample (H_2O) to clean the column and minimize the carryover.

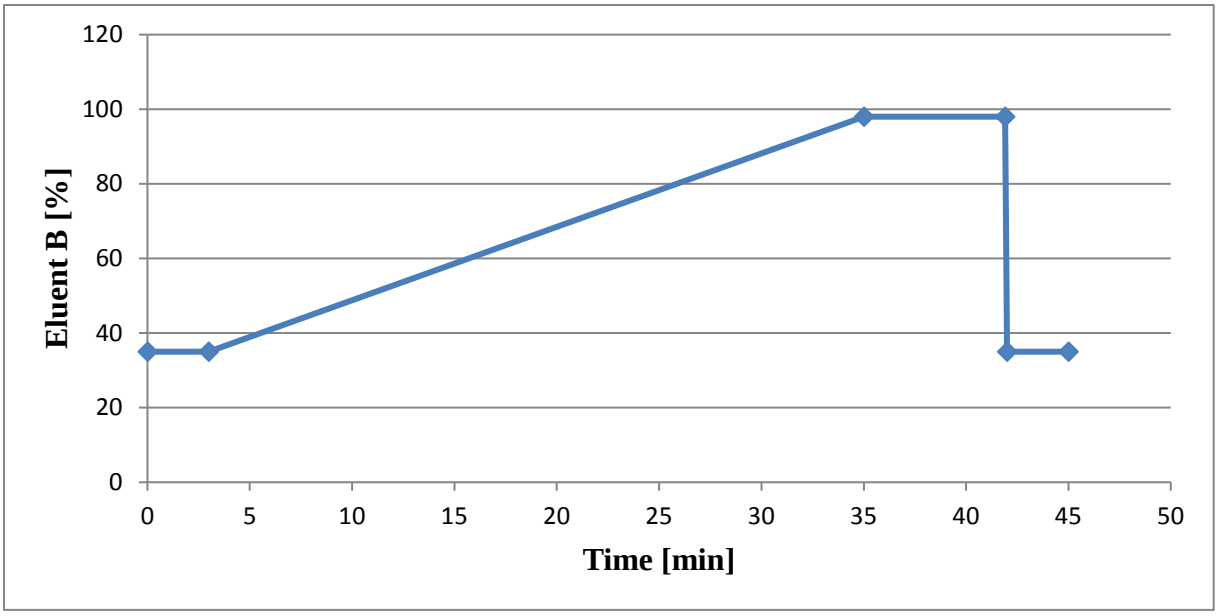

Figure 10: Gradient of HPLC method used for eicosanoid separation.

Time [min]	Eluent B [%]
0	35
3	35
35	98
41,9	98
42	35
45	35

Table 2: Exact time and percentages of eluent B for eicosanoid separation. Figure 10 displays the resulting graph created from this table.

4.3.3. Mass spectrometry method

The analytes from the HPLC system were eluted directly into the ESI source of the Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). The ESI was used in negative ionisation mode. The first quadrupole selected the ions with a m/z value between 250 and 750 and sent them to further for the analysis. First the Orbitrap performed a full scan of all arriving ions, which led to the creation of the MS^1 spectra. Additionally the six most abundant peaks were selected and sent to the collision cell, where they were fragmented with 30 eV to create the MS^2 spectra of the corresponding peaks. To further improve the analysis and allow the analysis of lower abundant peaks, a dynamic exclusion list was used. This list saved previously fragmented m/z values and did not allow the selection of these for the next four seconds. Additionally an inclusion list was used that

prioritizes the most common eicosanoid masses for the fragmentation selection. A small part of the detailed settings of the MS/MS analysis are listed in Table 3.

Overall method settings	
Method duration	45.00 min
Chromatographic peak width (FWHM)	6 s
Lock mass	344.9791 m/z
Lock mass tolerance	10.0 ppm
Inclusion	200.0 ppm
Polarity	Negative
In-source CID	0.0 eV
Full MS settings	
Resolution	35 000
Scan range	250-750 m/z
MS^2 settings	
Resolution	17 500
Scan range	200-2000 m/z
Isolation window	1.0 m/z
Intensity threshold	4.0e1

Table 3: Cut-out of the detailed MS/MS settings for the eicosanoid analysis.

4.4. Identification and quantification of eicosanoids

The identification and quantification of the eicosanoids was done with a bioinformatic and manual approach.

The analysed samples were searched against the lipid maps structure database to get a first idea of the contained eicosanoids. The first criterion in this step was mainly the exact mass which was searched for corresponding entries in the database using a group-made software. When a mass was found that related to a known biological active eicosanoid the second criterion was the pattern of the MS/MS fragmentation. The fragmentation was again compared to the entries in the lipid maps database and searched for some main points that had to be fulfilled. The three main peaks that are shown in the database had to be identical to the peaks in the MS/MS fragmentation in the mass of course but as well in the relative intensities

to each other. Although the intensities were not taken as the strictest criterion since some samples revealed all the peaks shown in the database but with different intensities. This is not too surprising since all the entries of the database were made in different laboratories on different systems which might have some influence on the fragmentation, especially when it comes to the intensities. Figure 11 shows the two mass spectra derived from the samples and the database and the three highest peaks are marked.

Since the concentration, especially in the not activated samples, were quite low it was not always possible to find the MS/MS pattern relevant for the metabolite in all of the samples. In this case the retention time, in correlation to the internal standards, and the exact mass was used to find the relevant peak and identify the metabolite. The retention time had to be similar in correlation to the internal standards within a range of three times the peak width of the analysing peak. However the MS/MS pattern had to be at least in one of the samples to ensure a correct identification. Figure 14 shows the comparison of a metabolite in two different samples in correlation to the internal standard.

After definite identification of the eicosanoids the corresponding peaks were searched in all samples and the area under the curve was determined. Furthermore, the area of the internal standards were quantified and used for the internal calibration. This ensures a minimisation of the random mistakes either happening during sample preparation or in the HPLC-MS/MS system and therefore allows a quantitative statement.

5. Results and discussion

5.1. Identification of the eicosanoids

In order to research a potential change in the releasate of the platelets four patients with different stages of metastatic melanoma were compared with one healthy donor. The platelets were collected freshly and processed as described above.

The identification of the eicosanoids in the samples worked well although some samples showed better fragmentation than other samples. Figure 11 shows the fragmentation pattern of Hepoxilin B3 on the left derived from the analysis and on the right side as shown in the lipid maps database. Highlighted in red are the three most abundant peaks. Although their intensities are not perfectly identical it nevertheless allows a definite identification of the eicosanoid.

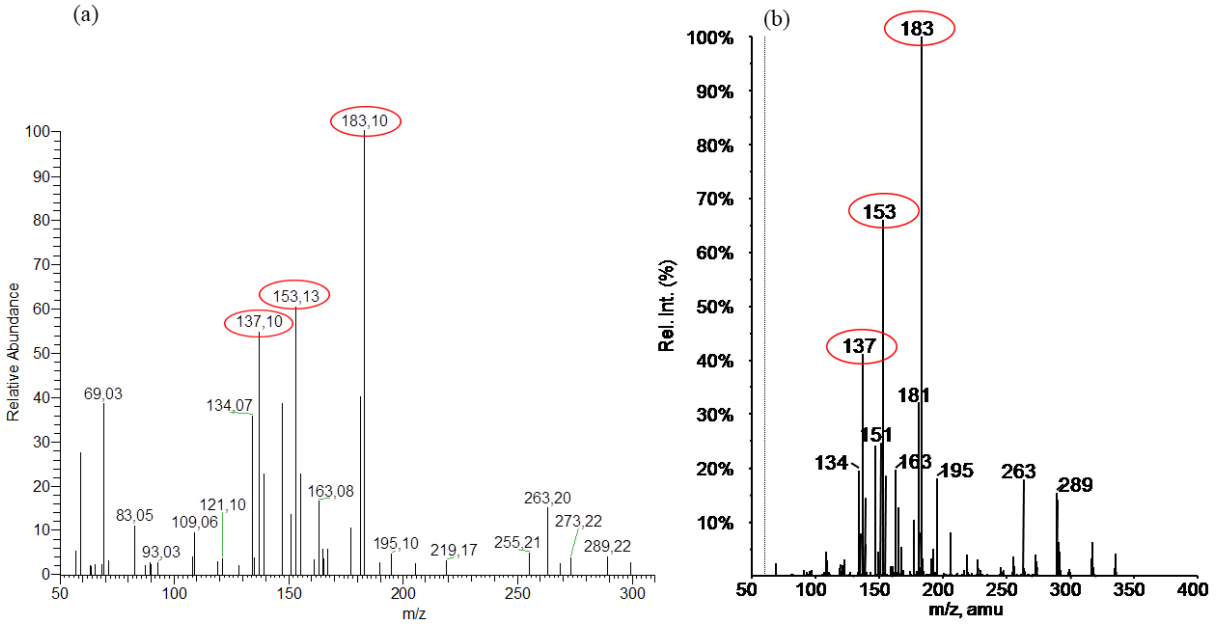

Figure 11: MS/MS fragmentation of Hepoxilin B3. (a) MS/MS from the analysis of patient 1 after 3 hours incubation time. The precursor mass of 335.18 m/z was taken at retention time 18.04 min. (b) Fragmentation pattern of Hepoxilin B3 from lipid maps structure database. Highlighted in red can be seen the three fragments with the highest intensities. Both spectra show the same pattern concerning these criteria but are also similar when analysing the lower abundant masses. The two spectra are almost identical which ensures the identification.

Some analytes on the other hand show a high isobaricity and structural similarity which often implicates a resemblance in the fragmentation pattern. In this case the differences are not in the most abundant peaks but in the lower abundant and have to be distinguished with those which nevertheless allows an exact identification. The HETEs are an example for eicosanoids

that only differ in the position of their hydroxyl group and therefore tend to show related fragmentation.

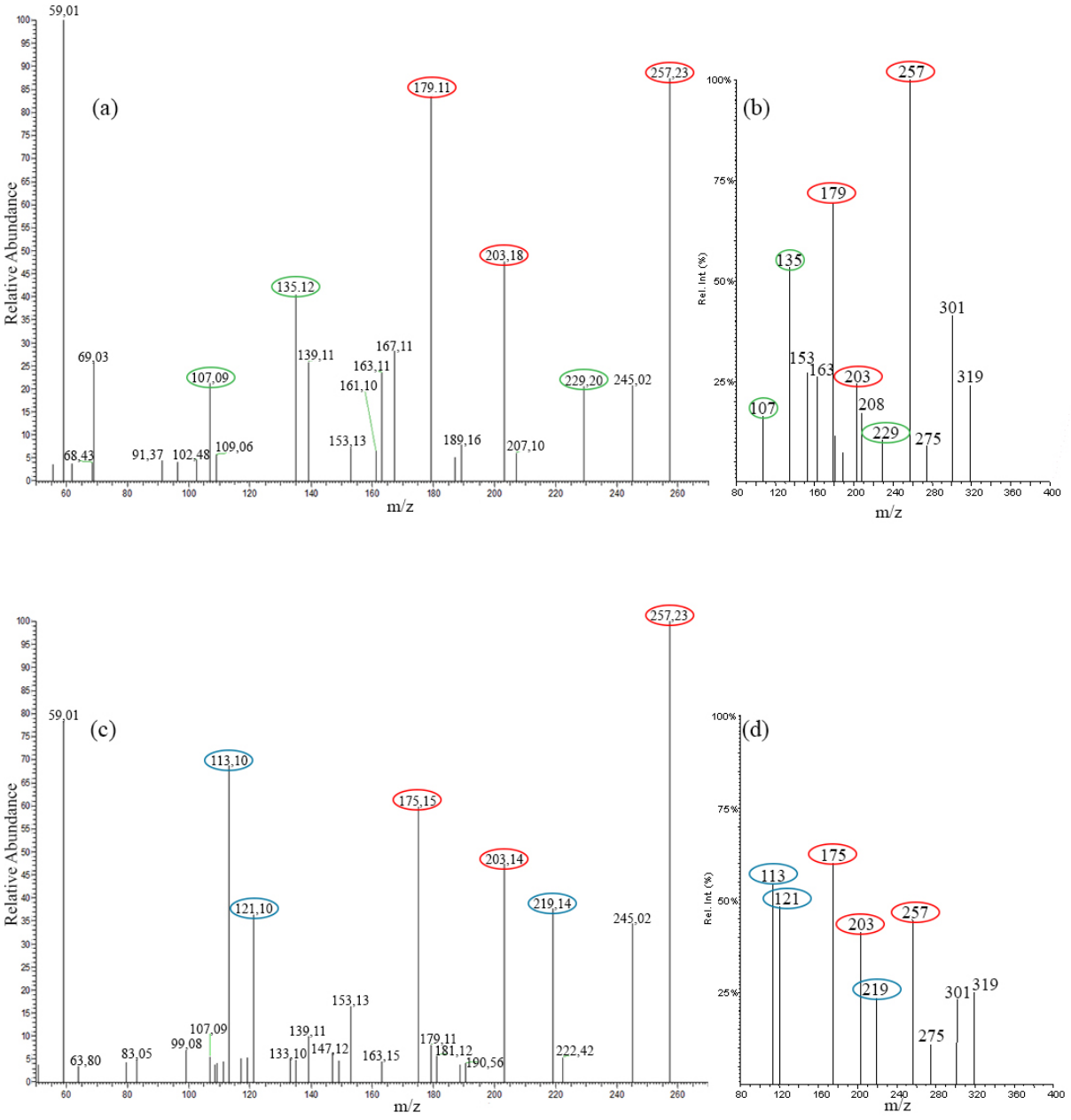

Figure 12: Comparison of the MS/MS spectrum recorded from the samples and provided by the lipid maps database of 15S-HETE and 12R-HETE. Spectrum (a) is recorded from patient 1 after 3 hours incubation with ionomycin at a retention time of 21.14 min and a precursor mass of 319.22. (b) is the corresponding fragmentation pattern of 12R-HETE as derived from the database. (c) is the spectrum of patient 1 after 3 hours incubation time at a RT of 20.75 min and a precursor mass of 319.22. (d) again is the corresponding spectrum from the database, representing 15S-HETE.

Figure 12 shows the MS/MS spectra of 15S-HETE and 12R-HETE. Highlighted in red are three of the most abundant masses that are detected after fragmentation. These three are

detectable in the spectra from the platelet samples (left) as well as the references from the database (right). According to these three masses a clear distinction in not possible, since the intensity is not representable as well. As mentioned before the exact identification is still possible when comparing the lower abundant masses. For the 12R-HETE above the green circled fragments and for the 15S-HETE below the blue highlighted masses are those of the unique fragments. The 15S-HETE is the only MS/MS that shows these masses and therefore clearly identified. The masses that are unique for the 12R-HETE on the other hand are also visible in the spectrum of the 15S-HETE, although in very low abundance.

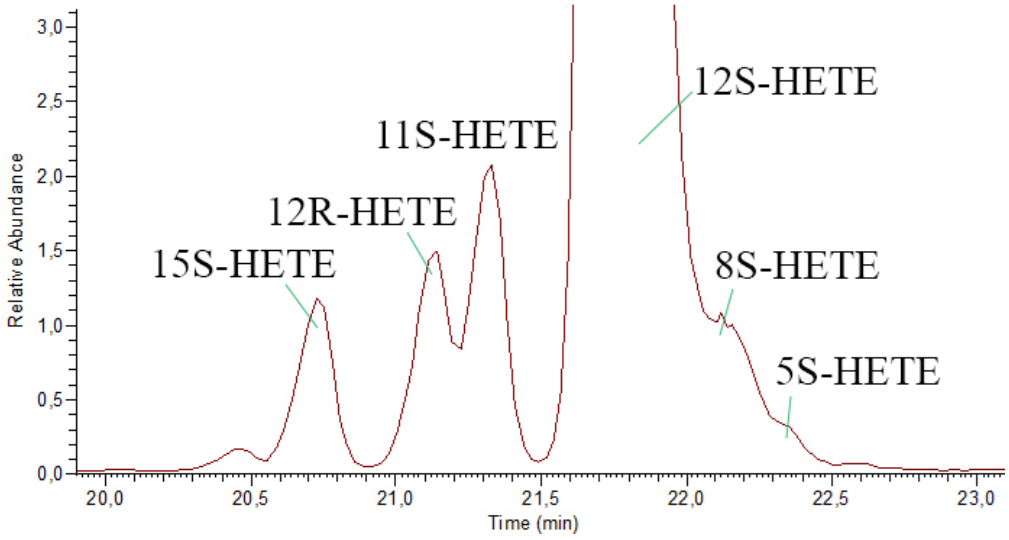

Figure 13: Extracted-ion chromatogram of 119 m/z with a zoom on the peak around time 21,79 (see Figure 14). The identified HETEs are written above the corresponding peaks.

These impurities of the 15S-HETE spectrum are easily explained when examining the chromatogram of the samples. In this special case the extracted-ion chromatogram (XIC) of 119 m/z which is the expected mass of the HETEs. Figure 13 shows a zoom of the peaks that were identified as HETEs. Considering the low structural differences that all the HETEs share the separation worked very well, although there is some place for improvement. As there can be seen some of the peaks are not completely baseline separated which makes the identification of the single peaks sometimes very challenging. Furthermore, the MS/MS fragmentation patterns show not such a high purity. As mentioned before the 15S-HETE shows some fragments that should not occur during its fragmentation but more during the fragmentation of 12R-HETE. The chromatogram in this case also shows a good separation of 15S and 12R-HETE. But the peak earlier tends to overlap a little bit with the 15S-HETE and therefore can cause the observed contaminations. This peak could not be identified since no

MS/MS pattern was recorded. Nevertheless it is very probable that it also belongs to the HETE subclass due to the mass and the time proximity of its elution. If it may be another natural HETE, an isomer or artefact produced during sample preparation cannot be said without MS/MS fragmentation.

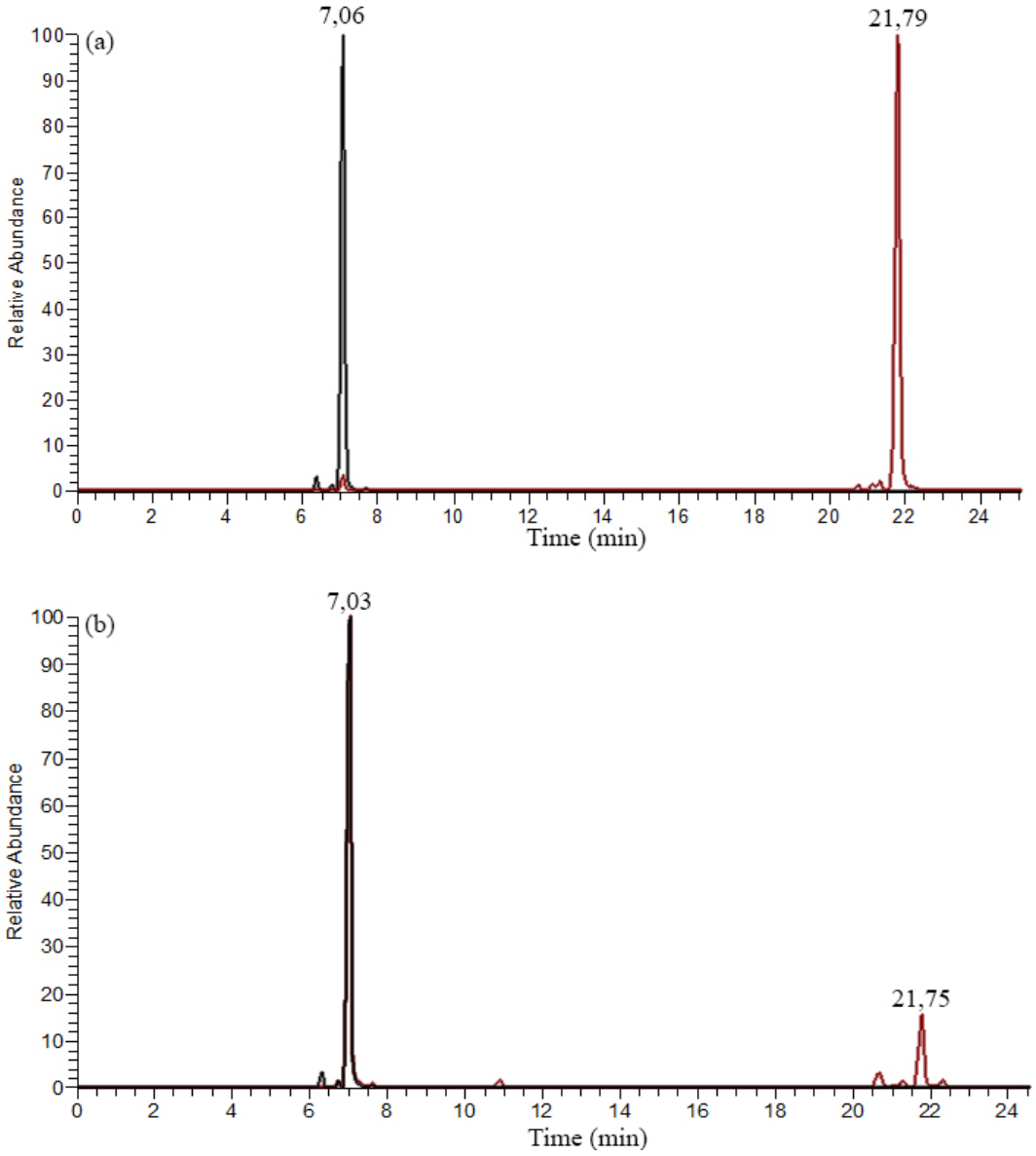

Figure 14: Overlay of the extracted-ion chromatograms (XIC) of the m/z values 355 (black) and 319 (red). Chromatogram (a) shows the XIC of the sample after 3 hours incubation and (b) the chromatograms for the control sample. The relative abundances are always compared to the highest peak and therefore a little bit misleading since they indicate the same value for the peaks of picture (a). Nevertheless it is visible that the metabolite with m/z of 319 (red line) shows significantly lower abundances in the control sample. This made it impossible to acquire a MS/MS fragmentation pattern for this sample. Nevertheless the difference in the retention time is the same as compared to picture (a)

where the MS/MS spectrum was recorded and correlated to 12S-HETE. And therefore the peak in chromatogram (b) could also be identified.

The XIC in Figure 14 shows the reliability of the chromatographic method and its stability. The retention time of the peaks that are shown, are nearly identical and therefore allow an exact identification of the metabolites even if an MS/MS fragmentation is not possible due to low amounts of analytes like in the control samples. The method showed in all samples and for all metabolites a similar picture without any great offsets in the chromatogram which allowed a stable analysis of even the lowest abundant eicosanoids.

With the criteria mentioned above, namely the exact mass, a MS/MS fragmentation pattern that undoubtedly identifies a metabolite in at least one sample and the stable retention time, it was possible to identify 28 different eicosanoids from different subclasses and biological pathways with very high certainty. Furthermore, 8 additional eicosanoids have been identified with lower certainty. The low confidence identifications mostly come from an insufficient or completely missing MS/MS fragmentation. The possibility that these eicosanoids are anyway identified in the sample arise from the exact masses that were found and could not belong to any other eicosanoid and are probable to find in the samples since some pathways depend on it. Table 4 shows a list of the 28 certainly found identified eicosanoids.

Table 5 shows the additional 8 eicosanoids which could not be identified with high certainty and therefore were not used for the further analysis. In the most cases the exact identification via MS/MS fragmentation pattern was not possible since the acquired MS/MS spectrum was full of impurities but nevertheless showed certain fragments of eicosanoids. Another point that strengthens the probability of the lipid in the sample is that all of them show a relation to pathways to eicosanoids that have been identified as seen in figure 15. The found HPETEs for example are precursor lipids of the HETEs that were surely identified in all the samples. Furthermore, a Hepoxilin-like eicosanoid was found. This lipid shows a very similar fragmentation pattern to Hepoxilin B3 with the same precursor mass but a different retention time. Since no appropriate MS/MS spectrum could be found in the database a clear identification was not possible. For the PGH_2 the identification is not possible because the MS/MS spectrum looks very similar to PGE_2 and PGD_2 and therefore might be one of these. Nevertheless PGH_2 is the precursor eicosanoid of many of the other PGs that were identified and therefore the probability that PGH_2 is present in the sample is quite high.

Identified Eicosanoids - Systematic Name	Synonym	MW
12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid	12S-HHTrE	280,2038
13R-hydroxy-9Z,11E-octadecadienoic acid	13(R)-HODE	296,2351
5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid	EPA	302,2246
(+/-)-12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid	12-HEPE	318,2195
12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid	12-oxo-ETE	318,2195
15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	15S-HETE	320,2351
12R-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	12R-HETE	320,2351
11S-hydroxy-5Z,8Z,11E,14Z-eicosatetraenoic acid	11S-HETE	320,2351
12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	12S-HETE	320,2351
8S-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid	8S-HETE	320,2351
5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	5S-HETE	320,2351
9S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	9-HETE	320,2351
11R-hydroxy-12E,14Z-eicosadienoic acid	11R-HEDE	324,2664
9-oxo-15S-hydroxy-5Z,10Z,13E-prostatrienoic acid	PGA2	334,2144
8-hydroxy-11S,12S-epoxy-5Z,14Z,9E-eicosatrienoic acid	Hepoxilin A3	336,2301
10-hydroxy-11S,12S-epoxy-5Z,8Z,14Z-eicosatrienoic acid	Hepoxilin B3	336,2301
(+/-)-14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid	(+/-)-14-HDoHE	344,2351
(+/-)-4-hydroxy-5E,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid	(+/-)-4-HDoHE	344,2351
9,15-dioxo-11R-hydroxy-5Z,13E-prostadienoic acid-cyclo[8S,12R]	8-iso-15-keto-PGE2	350,2093

9-oxo-11S,15S-dihydroxy-5Z,13E-prostadienoic acid	11β-PGE_2	352,2250
9-oxo-11R,15S-dihydroxy-5Z,13E-prostadienoic acid	PGE_2	352,2250
9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoic acid	PGD_2	352,2250
5S,12R,20-trihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	20-hydroxy LTB_4	352,2250
9,15-dioxo-11R-hydroxy-5Z-prostenoic acid	13,14-dihydro-15-keto-PGE_2	352,2250
9S,11S,15S-trihydroxy-5Z,13E-prostadienoic acid	11β-PGF_2	354,2406
9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid-cyclo[8S,12R]	8-iso-$PGF_{2\alpha}$	354,2406
9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid	TXB_2	370,2355
9S,11R,15S-trihydroxy-17-phenyl-18,19,20-trinor-5Z,13E-prostadienoic acid	17-phenyl-trinor-$PGF_{2\alpha}$	388,2250

Table 4: Identified eicosanoids in the patient and healthy samples.

Figure 15 was created to give a short overview of all the eicosanoids that have been found in the samples and how they are related in terms of biological pathways. Most of the detected eicosanoids are, as expected, derived from arachidonic acid. And within these metabolites the HETEs and PGs are the most present, but also hepoxilins, leukotrienes and thromboxanes were identified. Thromboxane A_2 as one of the known players in platelet aggregation could not be identified which is not very surprising since it is known to be metabolised very fast to create TXB2 which, on the other hand was identified in all the samples.
Additional to the AA derived metabolites a few have also been identified that are metabolised from eicosapentaenoic acid (EPA) which has also been found in the samples.

Identified Eicosanoids - Systematic Name	Synonym	MW
	Hepoxilin-like	336,2301
5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	LTB_4	336,2301
156S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	15(S)-HpETE	336,2301
12S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	12(S)-HpETE	336,2301
5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	5(S)-HPETE	336,2301
9S,11R-epidioxy-15S-hydroxy-5Z,13E-prostadienoic acid	PGH_2	352,2250
9-oxo-5Z,10,12Z,14E-prostatetraenoic acid	15-deoxy-PGA_2	316,2038
(+/-)-11-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid	11-HEPE	318,2195

Table 5: 8 eicosanoids that have been partially identified. All of the eicosanoids show MS/MS fragmentation but either not pure enough to allow a correct identification or have no corresponding database entry.

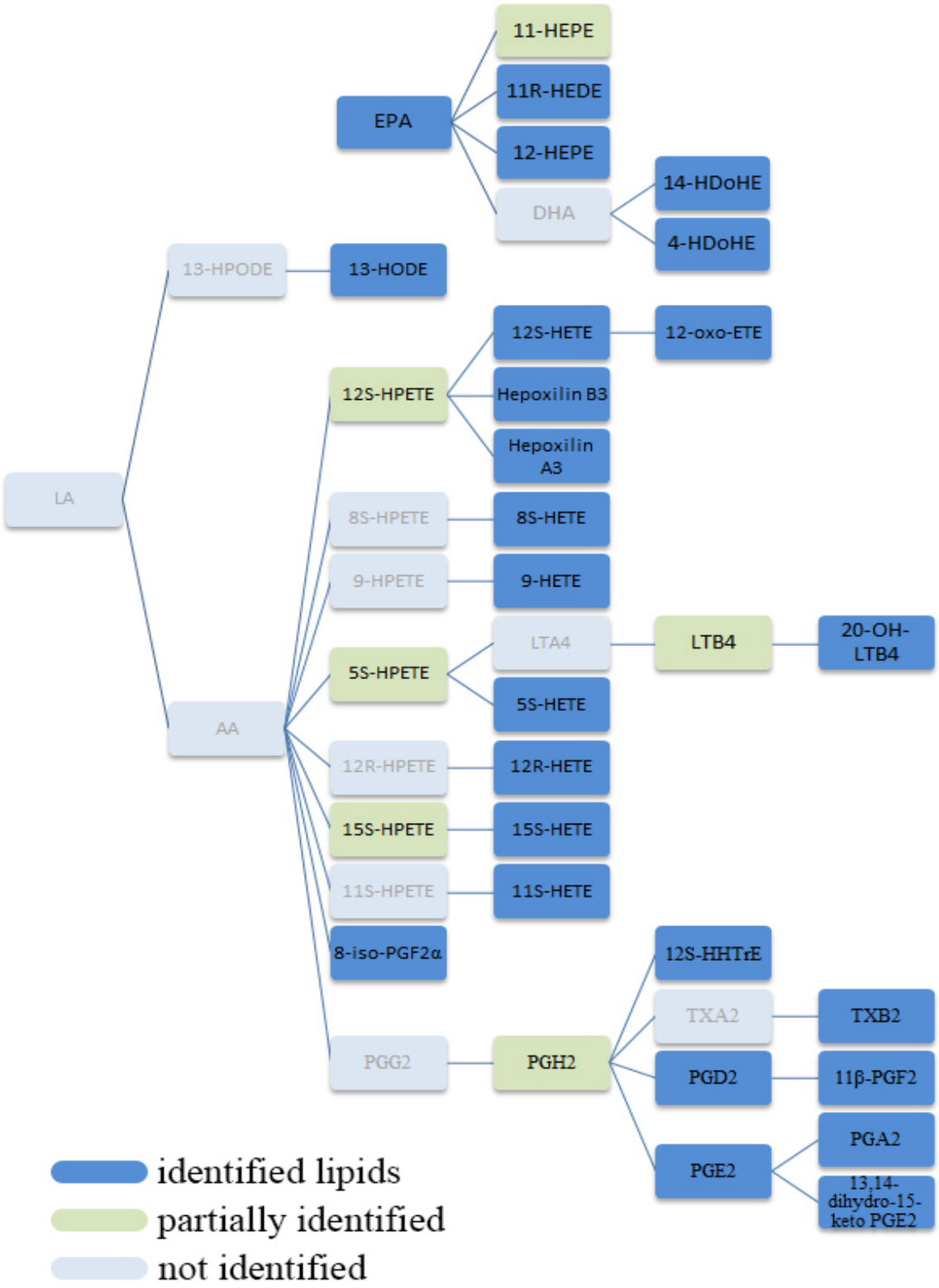

Figure 15: Pathways that lead to eicosanoid production based on eicosanoids found in samples. The colours indicate the states that each lipid is found. Dark blue shows one of the definitely identified eicosanoids as shown in Table 4, light green shows the lipids whose identification is not certain and light blue shows lipids that have been formed during the biosynthesis of the identified but have not been detectable.

5.2. Relative quantification and comparison of the donors

In order to recognize a difference in the behaviour of the platelets of the patients and the healthy donor the samples incubated for 15 minutes and three hours were compared to the control sample. Hence all the peaks belonging to clearly identified eicosanoids were analysed and the area under the curve was calculated. The values obtained from the control samples were taken as base and the other values were correlated to those in order to get a relative quantification as fold change.

The quantification shows from an analytical point of view that the difference between 15 min and 3 hours of incubation can be quite high. In some cases, as suggested before, this makes the difference between being able to identify the metabolite via MS/MS fragmentation or not. On the other hand the changes are not identical for all the analytes. Within the samples the differences of the individual analytes can be quite big. Figure 16 shows the fold change of three different analytes as seen in the samples of patient 1. The graph shows that the change of 12S-HHTrE is insignificant and the longer incubation did have nearly no effect. For the 12R-HETE the difference between 15 minutes and 3 hours incubation is much larger. The biggest effect in this case is seen for the Hepoxilin B3, whose amount changes from about 14 fold change to nearly 80 fold change. As proposed before, most of the analytes behave like 12R-HETE and Hepoxilin B3 which shows the importance of the 3 hours incubation.

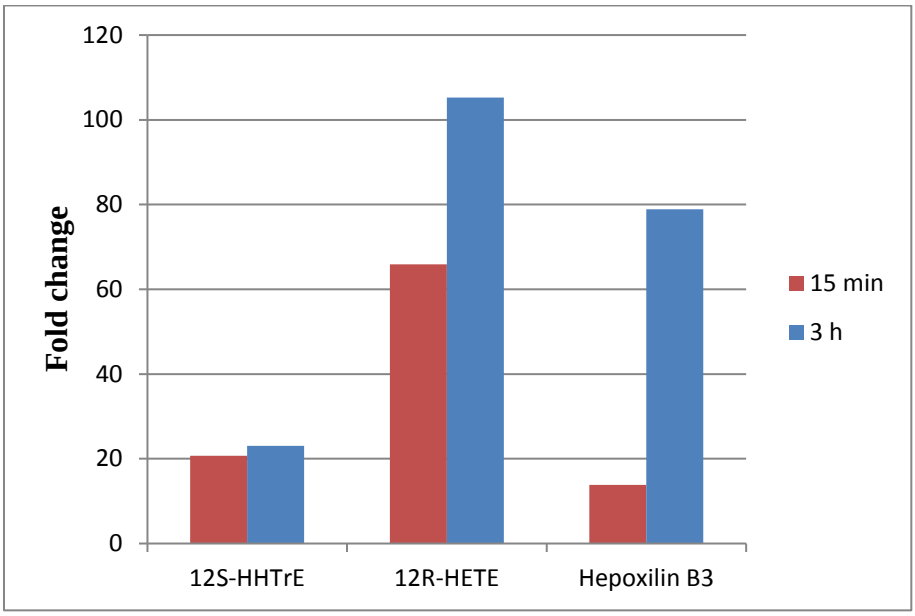

Figure 16: Difference of fold change of three examples from patient 1. The red bar shows the fold change of the sample with 15 min incubation time and the blue bar after 3 hours incubation.

One of the most obvious differences that could be detected was the missing of some metabolites in certain samples. As shown in figure 13, 8S-HETE and 5S-HETE are detected after three hours incubation for patient 1. Although the separation in this case did not work as well as for others the identification was very certain but the quantification was quite challenging in order to get a comparable result. 8S- and 5S-HETE were found in all samples from the melanoma patients and the peaks were more or less quantifiable. But for the healthy donor these eicosanoids were not detectable at all but instead 9-HETE was found, which on the other hand could not be detected in the patient samples.

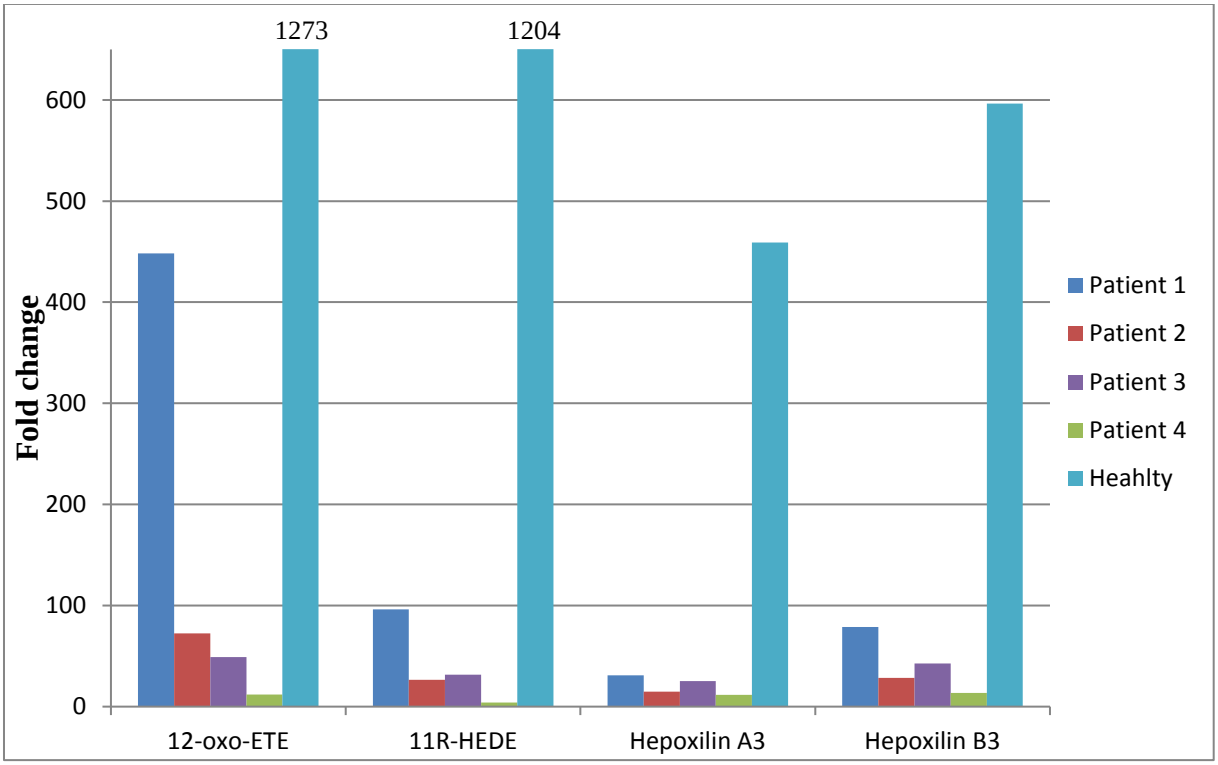

Figure 17: Fold change values of four examples with the highest change after 3 h incubation. The four examples show the highest changes within the different donors. The fold change for the healthy platelets is not displayed correctly for 12-oxo-ETE and 11R-HEDE since the amounts are way too high. The correct value is written above and nearly double the amount presented here.

Aside from these small differences in the composition of the healthy and diseased platelets the main difference is the amount of eicosanoids that is produced during the activation. The amount of eicosanoids measured in the control samples for a distinct metabolite is quite similar in all found donors. This means that the basis for the comparison of the fold changes is more or less the same for all samples. After the incubation, as well for 15 min but more obvious for three hour incubation, there are pretty big differences. Figure 17 displays the fold

change values for four examples that show the highest difference when comparing the healthy donor with the patients. As shown is the fold change of the healthy donor compared to all four patients by far the highest. The 12-oxo-ETE, which shows the highest fold change for all the identified eicosanoids in the healthy donor is significantly lower for all the patients and depicts the highest difference. But also the 11R-HEDE and the Hepoxilins show a clearly significant change in the levels of expression when comparing the healthy with the diseased donors.

The metabolites shown in figure 18 display a much lower change since the healthy values are not nearly as high as in the eicosanoids presented in figure 17. Nevertheless, the difference to the patient derived platelets is obvious. Furthermore, when examining both graphs 17 and 18 closely, there can be suspected to be a certain pattern that is typical for a patient. This is believed to be correlated to the stage that the diseased donor currently is in. Noticeable for example is that patient 4 shows by far the lowest fold change for nearly all the metabolites. Patient 1 on the other hand most of the time shows a quite high expression compared to the patients, although it is still much lower than compared to the healthy donor. Patient 2 and 3 show an expression pattern that is located somewhere between patient 1 and 4 in the means of the fold change values.

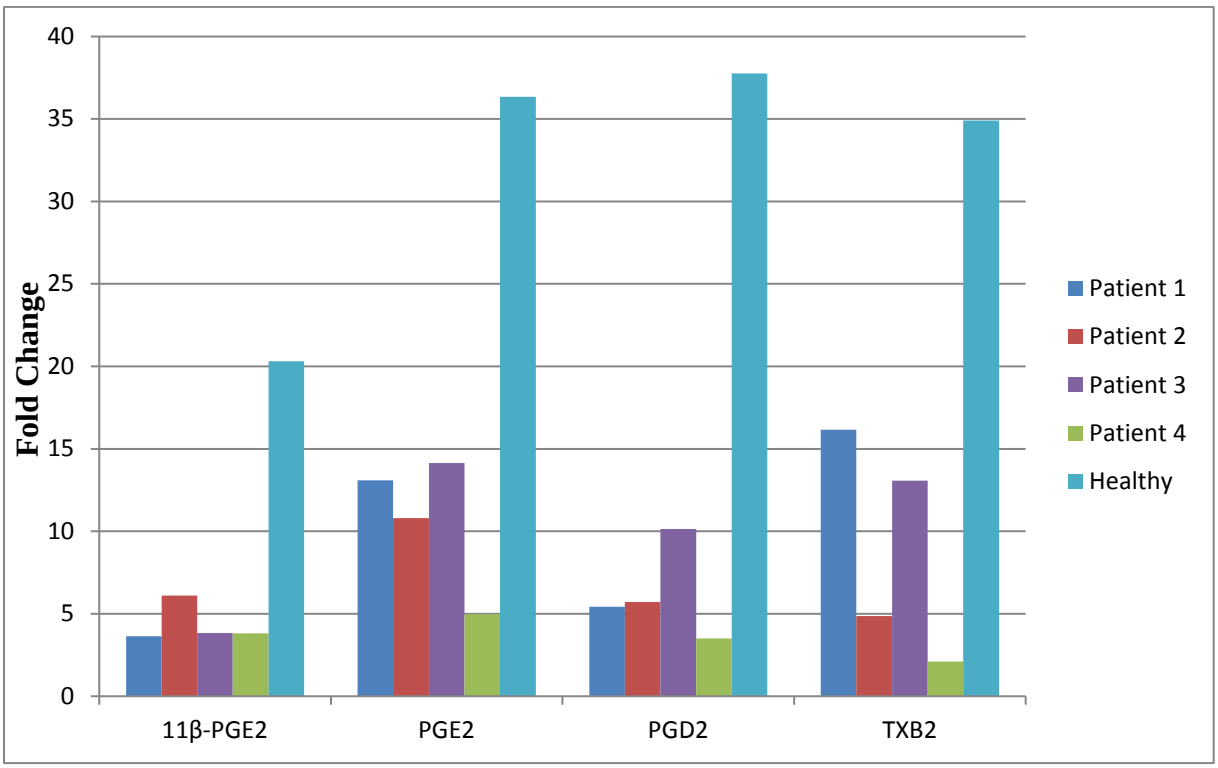

Figure 18: Fold change values of four metabolites from all donors after 3 hours incubation time.

The difference in the expression levels of patient 1 and patient 4 becomes more evident when regarding figure 19. This displays the direct comparison of the fold change amounts from these two patients. As clearly seen, patient 1 shows very high HETE levels. When comparing the highest fold change of patient 1, the 12-oxo-ETE with an almost 450 fold change and the highest value of patient 4, the EPA with 32 fold change the difference becomes even more impressive. For most of the PGs the difference is not that apparent and sometimes they even show the same levels of eicosanoid expression. Nevertheless, the difference that these diseased donors show is very obvious and clearly enables a distinction of these two, and of course also to the healthy donor.

An also very interesting observation is the pattern that is shown by the healthy donor. A few metabolites that are very high regulated, many that show a medium regulation, and some that show little regulation. This pattern is not so apparent for the patients and gets more and more adjusted to the same levels of fold change. As Figure 19 displays, patient 1 shows a high regulation of some metabolites and a medium and low fold change of many. Patient 4 losses this pattern completely and only shows a low regulation for all its eicosanoids.

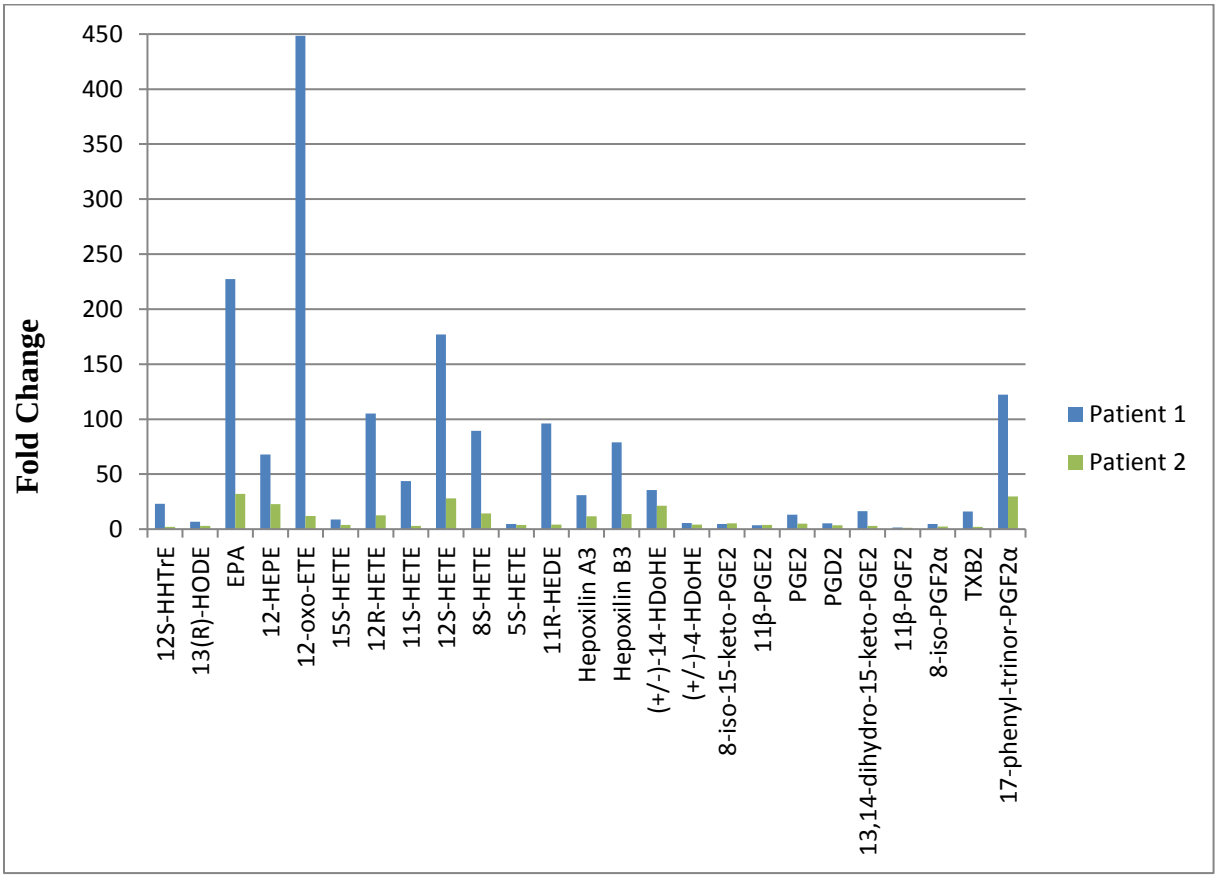

Figure 19: Comparison of the fold change from patient 1 and patient four.

These comparisons clearly show that there is an apparent difference in the eicosanoid levels between patients and the healthy donor, but more important also a difference between the patients. The discussed differences in the fold change for all five donors undoubtedly show that the high throughput method presented in this work is capable of displaying the eicosanoid levels in quite short time.

6. Conclusion and perspective

With the development and advances for the treatments for several diseases the life expectancy of an average human is today higher than ever before. But with the longer life and lower probability to die of an infection due to the good working antibiotics, cancer has become one of the major causes of death [51]. Therefore, it is not surprising that enormous efforts are taken in order to treat and recognize this disease more effectively. But especially when considering the molecular mechanisms that influence cancer development or the impact that a tumor growth has on the whole system, the knowledge is quite incomplete. Although every cancer has its basis in a mutation of the genome of a cell, the changes in protein expression and metabolite levels cause the apparent cancerous behaviour. Therefore, in order to treat cancer, these molecular mechanisms have to be studied.

Melanoma as a very aggressive form of skin cancer, which is influenced by many environmental factors that are sometimes not avoidable, was the main interest for this work [10]. Strictly speaking the blood platelets of patients diagnosed with malignant melanoma. These blood platelets have been shown to play certain roles in the development and furthermore the metastasis of several cancer types [19,20]. But there is also evidence, that the platelets themselves change during the progression of tumorogenesis [23].

To research the eicosanoid expression of the platelets they have been derived freshly from patients' blood, purified and activated using ionomycin. The releasate of the platelet suspension has been taken to investigate the expression changes in the eicosanoid level. In order to achieve this, the supernatant was first mixed with cold ethanol to precipitate the proteins. The lipids in the liquid phase were extracted using a solid-phase extraction. The eicosanoids were analysed in an HPLC-MS/MS system and scanned for the lipids of interest. The obtained data was processed manually and the expression levels in the metabolites were compared.

The analysis showed very high potential. 28 Eicosanoids from different pathways, derived from arachidonic acid and EPA, could be identified with high certainty. Furthermore, 8 additional lipids were identified with lower certainty, but nevertheless are very probable to be in the sample. The measured eicosanoids include HETEs, PGs, thromboxane, hepoxilins and a few others. These were compared semi quantitative and the expression levels without ionomycin, after 15 minutes, and after three hours of incubation were determined. The single lipids themselves showed very high differences in the fold change. Many of the HETEs show a very high change after incubation whereas many of the PGs do not show high differences,

but nevertheless a definite variance to the not activated platelets. Far more interesting is the comparison of the fold change levels of the particular patients with one healthy donor. A unique pattern for all of the patients and the healthy donor as well is evident. The biggest difference can be seen in the expression levels of the HETEs. While they do not show a significant difference in the amount for the not activate platelets they are quite dissimilar after the three hours of incubation. The healthy patient shows an extreme increase of the fold change of to up to 1200 for the highest, and several hundred for many others. The patients on the other hand show eicosanoid levels that are much lower than this. Patient 1 is for most metabolites the one with the highest fold change and patient 4 shows very low changes even for the three hour incubation time. This is an indicator for the exhaustion of the platelets during the progression of the disease.

The data shown in this work indicates that there is a correlation between a different behaviour of the platelets and the stage of the melanoma disease. But what has been shown beyond doubt is that the analytical method used for the analysis of blood platelets is capable of displaying even small differences for certain eicosanoids. The analytical method has of course possibilities to enhance. While the HPLC-MS/MS method, the SPE and the platelet activation work properly with some smaller improvement potentials the biggest drawback of the method is the manual evaluation of the data. Software that is available for proteomics is not offered in such great amounts for the research in lipidomics and metabolomics. These software packages, which ease and accelerate the comparison of the data, are today in development will make analysis of this manner much faster.

As the data presented in this work already suggests, there is indeed a significant difference between the eicosanoid levels of the healthy donor compared to the patients. To assure these findings and furthermore be able to create a biological assumption a bigger study has to be performed that includes more patients of different stages of the disease and more healthy donors. Furthermore, the influence of treatments for melanoma and also the influence of other cancer types could be investigated in order to exactly define the change of the platelet behaviour during cancer diseases.

7. Abstract

In order to achieve a better understanding of biological systems per se but especially in correlation with different severe diseases like cancer genomic, transcriptomic, proteomic, lipidomic and metabolomic methods are today widely used for research. But not only the processes that influence the development and progression of tumors are interests of research but also how the cancer affects the whole system. The blood platelets are described to influence cancer and on the other hand are changed by cancer themselves [5].

This work presents an analytical method that allows the analysis of the releasate pattern of eicosanoids of blood platelets. Therefore blood platelets of four patients diagnosed with melanoma and one healthy donor were derived and purified. The platelets have been activated with ionomycin in order to achieve the production and release of eicosanoids. The proteins of the supernatant were precipitated and the eicosanoids purified with a solid-phase extraction. The lipids were analysed using a HPLC-MS/MS system and the data was evaluated manually.

With this method 28 eicosanoids including HETEs, PGs, thromboxane, hepoxilin and others could be identified and quantified. Additional 8 eicosanoids were identified with lower certainty but are believed be in the sample as well. The lipids were quantified and the values of the control samples were compared to the ones of the platelets activated for 15 minutes or three hours. This showed, on the one hand, a difference between the distinct subclasses of eicosanoids. The HETEs for example showed a very high fold change whereas the PGs showed very little change. On the other hand, there was a visible difference between the patients and the healthy donor. Overall the diseased platelets showed a much lower fold change than the healthy platelets, which could be an indicator for an exhaustion of the platelets and a change in the activity in different stages of the disease.

8. Zusammenfassung

Um ein besseres Verständnis biologischer Systeme vor allem in Zusammenhang mit schweren Krankheiten wie Krebs zu erreichen, werden heute Genomic, Transcriptomic, Proteomic Lipidomic und Metabolomic Methoden in der Forschung verwendet. Aber nicht nur die Prozesse die zur Entwicklung von Tumoren führen sind dabei interessant, sondern auch wie eine Krebserkrankung das ganze System beeinflussen kann. Blutplättchen können einerseits den Verlauf einer solchen Erkrankung beeinflussen andererseits gibt es aber auch Hinweise darauf, dass sie selbst im Laufe einer schweren Krankheit verändert werden.

Diese Arbeit stellt eine analytische Methode vor, welche es ermöglicht die bei Aktivierung freigesetzten Eicosanoide von Blutplättchen zu analysieren. Dafür wurden Blutplättchen von vier Patienten mit Melanom und einem gesunden Spender entnommen und gereinigt. Die Plättchen wurden mit Ionomycin aktiviert um eine Ausschüttung der Eicosanoide zu erreichen. Die Proteine des Überstands wurden ausgefällt und die Eicosanoide mit einer Festphasen-Extraktion gereinigt. Die Lipide wurden mittels eines HPLC-MS/MS Systems analysiert, und die Daten manuell ausgewertet.

Mit dieser Methode konnten 28 verschieden Eicosanoide unter anderem HETEs, PGs, Hepoxilin und Thromboxan identifiziert werden. Acht weitere Eicosanoide konnten mit geringerer Sicherheit identifiziert werden, werden aber dennoch in den Proben vermutet. Die Lipide wurden quantifiziert und die Werte der Kontrollproben mit denen der 15 Minuten und drei Stunden aktivierten verglichen. Dies zeigt einerseits die Unterschiede zwischen den einzelnen Subklassen der Eicosanoide. Die HETEs zum Beispiel zeigen eine sehr starke Erhöhung nach drei Stunden wohingegen die PGs nur eine sehr geringe Erhöhung zeigen. Andererseits gibt es einen signifikanten Unterschied zwischen den Patienten und dem gesunden Spender. Insgesamt zeigen die Plättchen der erkrankten Spender eine sehr viel geringere Ausschüttung von Lipiden als die gesunden Blutplättchen, was ein Anzeichen dafür sein kann, dass sie die Plättchen in einem erschöpften Zustand befinden und ihre Aktivität unterschiedlich ist, je nachdem in welchem Stadium sich die Krankheit befindet.

9. References

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