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Secretome analysis of activated platelets – a proteomic and lipidomic approach

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# For my parents

"Curiosity killed the cat, but satisfaction brought it back."
(John Doe, *The Titusville Herald Newspaper* (23 December 1912), p. 6.)

"Nothing is impossible; science has taught us that."
(Christie, A. *Passenger to Frankfurt: An Extravaganza*; [Published for] the Crime Club [by] Collins: London, 1970.)


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All my friends that I was allowed to meet in my time at university and aside from it deserve my deepest appreciation for encouraging me, making me enjoy the best times and showing me the important things in life.

On a personal note I would like to express my deepest gratitude to my family, which never steers me wrong, their encouragement and for always making me feel loved and cherished.

Nothing of this would have been possible without my parents, who I count as my biggest blessing.

So Long, and Thanks for All the Fish!

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## List of Abbreviations

| | |
|---|---|
| 12S-HHTrE | 12-hydroxy-heptadecatrienoic acid |
| 13-HODE | 13-hydroxy-octadecadienoic acid |
| 2D-GE | two-dimensional gel electrophoresis |
| 2D-PAGE | two-dimensional polyacrylamide gel electrophoresis |
| AA | Arachidonic acid |
| ACN | Acetonitrile |
| APCI | Atmospheric Pressure Chemical Ionization |
| CID | Collision Induced Dissociation |
| $cPLA_{2\alpha}$ | cytosolic phospholipase A2 |
| EET | Epoxyeicosatrienoic acid |
| EI | Electron Impact |
| EPA | Eicosapentaenoicacid |
| ESI | Electrospray Ionization |
| FA | fatty acyl |
| FDR | false discovery rate |
| GC | gas chromatography |
| HETE | Hydroxyeicosatetraenoic acid |
| HPLC | High Performance Liquid Chromatography |
| IS | internal standards |
| LA | Linoleic acid |
| LC | liquid chromatography |
| LLE | liquid liquid extraction |
| LT | leukotrienes |
| MALDI | Matrix Assisted Laser Desorption Ionization |
| MS | mass spectrometry |
| NRP | neutral resting point |
| NSAID | non-steroidal anti-inflammatory drugs |
| PG | prostaglandins |
| PRP | platelet rich plasma |
| PTM | post translational modifications |
| PUFA | polyunsaturated fatty acids |
| RT | retention time |
| SCX | strong cation exchange |
| SPE | solid phase extraction |
| SPM | specialized pro-resolving mediators |
| TIS | total ion spectrum |
| TNF-α | tumour necrosis factors |
| TOF | time of flight |
| TPO | thrombopoietin |
| TX | thromboxanes |
| vWF | von Willebrand factor |
| XIC | extracted ion chromatogram |

## Abstract

Platelets are anuclear blood cells, which are involved in hemostasis and wound healing and seem to be involved in a lot of highly relevant diseases, including cancer, COVID and obesity. Upon activation, platelets may form and release a lot of potent molecules involved in various disease processes. Previous data indicated that platelets might actively synthesize proteins although their lack of a cell nucleus. This work aimed to identify bioactive molecules released from activated platelets and further investigated whether new synthesis of proteins was indeed possible. In addition, proteome profiling was complemented with eicosanoid profiling, as eicosanoids are also potent biomolecules involved in a large range of effects.

As platelets react highly sensitive to any disturbance in their environment it was necessary to develop a method for the gentle isolation of platelets avoiding premature activation. This allows the generation of suitable control samples in comparison to the activated ones. Blood samples of four healthy donors were collected and the platelets concentrated by differential centrifugation. One aliquot per sample was processed after one hour at rest. The other two aliquots per sample were activated with the ionophor ionomycin and collected 15 minutes and three hours after activation, respectively. The protein samples were digested and processed according to an established protocol. The lipids were extracted by solid phase extraction. To control the quality of the preparation and quantification, internal standards were used. The analysis was performed in technical triplicates, with a high resolution Orbitrap mass spectrometer. The evaluation of proteomics data was supported by bioinformatical tools. The lipidomics data evaluation required many more manual evaluation steps. The results again demonstrated that platelets are capable of synthesizing proteins and displayed positive and negative regulatory events of proteins in response to activation. The down-regulatory events may prevent an escalation of blood coagulation prohibiting unwanted thrombotic events. The search for signal lipids yielded 26 eicosanoids, 22 of which were found significantly upregulated and which could be assigned to different pathways. The molecules show pro- and anticoagulating properties. The results clearly indicated that the aim of preventing premature activation during platelet isolation was successfully achieved. Consequently, a large number of bioactive molecules released by platelets upon activation, comprising both proteins and eicosanoids, was successfully identified. Remarkably, several of

the identified molecules are known to have pro-survival functions. Thus, the functional implications of platelets in various diseases might have been rather under-estimated.

## Zusammenfassung

Thrombozyten sind kernlose Blutzellen, die an der Blutgerinnung und Wundheilung beteiligt sind und bei vielen hochrelevanten Krankheiten wie Krebs, COVID und Fettleibigkeit eine Rolle zu spielen scheinen. Nach ihrer Aktivierung können Thrombozyten eine Vielzahl von Molekülen mit hoher biologischer Wirksamkeit bilden und freisetzen, die auch an verschiedenen Krankheitsprozessen beteiligt sein können. Frühere Daten deuteten darauf hin, dass Thrombozyten aktiv Proteine synthetisieren können, obwohl sie keinen Zellkern besitzen. Ziel dieser Arbeit war es, bioaktive Moleküle zu identifizieren, die von aktivierten Blutplättchen freigesetzt werden, und weiter zu untersuchen, ob eine Neusynthese von Proteinen tatsächlich möglich ist. Eine Neusynthese von Proteinen würde das vielfältige funktionelle Repertoire von Plättchen gut erklären können. Darüber hinaus wurde das Proteom-Profiling durch ein Eicosanoid-Profiling ergänzt, da Eicosanoide ebenfalls potente Biomoleküle sind, die in extrem geringen Konzentrationen effektiv an einer Vielzahl von Wirkungen beteiligt sind. Da Thrombozyten sehr empfindlich auf jede Störung in ihrer Umgebung reagieren, war es notwendig, eine Methode zur schonenden Isolierung von Thrombozyten zu entwickeln, die eine vorzeitige Aktivierung verhindert. Dies ermöglicht die Herstellung geeigneter Kontrollproben im Vergleich zu den aktivierten Proben. Blutproben von vier gesunden Spendern wurden schonend, ohne Vakuumröhrchen durch freien Blutfluss aus der Vene in ein entsprechendes Probengefäß entnommen und die Thrombozyten durch Differenzialzentrifugations-Schritte konzentriert. Ein Aliquot pro Probe wurde nach einer Stunde Ruhezeit verarbeitet. Die beiden anderen Aliquote pro Probe wurden mit dem Ionophor Ionomycin aktiviert und 15 Minuten bzw. drei Stunden nach der Aktivierung weiterverarbeitet. Die Proteinproben wurden nach einem etablierten Protokoll verdaut und verarbeitet. Die Lipide wurden durch Festphasenextraktion extrahiert. Zur Kontrolle der Qualität der Identifizierung und Quantifizierung wurden interne Standards verwendet. Die Analyse wurde in dreifacher technischer Ausführung mit einem hochauflösenden Orbitrap-Massenspektrometer durchgeführt, der mit entsprechenden Hochleistungs-Chromatographie-Systemen gekoppelt waren. Die Auswertung der Proteomikdaten wurde durch bioinformatische Tools unterstützt. Die Auswertung der Lipidomics-Daten erforderte viele manuelle Auswertungsschritte.

Die Ergebnisse zeigten erneut, dass Thrombozyten in der Lage sind, Proteine zu synthetisieren, und positive und negative regulatorische Ereignisse von Proteinen als Reaktion auf die Aktivierung aufweisen. Tatsächlich konnten viele Wachstumsfaktoren wie etwa PDGFA, PDGFB und VEGFC und EGF neben zahlreichen weiteren regulatorisch aktiven Proteinen im Sekretom nachgewiesen werden. Die herunterregulierenden Proteine scheinen die Aufgabe zu haben eine Eskalation der Blutgerinnung verhindern um unerwünschte thrombotische Ereignisse unterbinden. Die Suche nach Signallipiden ergab 26 Eicosanoide von denen 22 durch Aktivierung im Überstand signifikant hochreguliert wurden und die sowohl koagulationsfördernden als auch koagulationsinhibierenden Effekten zugeordnet werden konnten. Für die Bildung dieser Eicosanoide waren hautsächlich Cycloxygenasen und Lipoxygenasen verantwortlich, und die wichtigsten Enzymsubstrate waren Arachidonsäure, Eicosapentaensäure und Linolsäure.

Die Ergebnisse zeigen deutlich, dass das Ziel, eine vorzeitige Aktivierung während der Thrombozytenisolation zu verhindern, erfolgreich erreicht wurde. Folglich konnte eine große Anzahl bioaktiver Moleküle identifiziert werden, die von Thrombozyten bei der Aktivierung freigesetzt werden und sowohl Proteine als auch Eicosanoide umfassen. Bemerkenswerterweise sind mehrere der identifizierten Moleküle dafür bekannt, dass sie überlebensfördernde Funktionen haben. Somit ist es plausibel, eine auch tumorpromovierende Funktion anzunehmen, die auch mit der biologischen Aufgabe der Wundheilung kompatibel ist. Die funktionelle Bedeutung von Blutplättchen bei so wichtigen Krankheiten wie etwa Krebs kann daher kaum überschätzt werden.

## Secretome analysis of activated platelets – a proteomic and lipidomic approach

## 1. Introduction

The cornerstone of what should become this master thesis was laid years ago by Professor Gerner as he was researching another topic. What caught his eye was the appearance of new spots in a 2D-SDS PAGE of activated platelets (see Figure 1). A hypothesis was formulated that platelets synthesize new proteins upon activation. This work tries inter alia to investigate this claim.

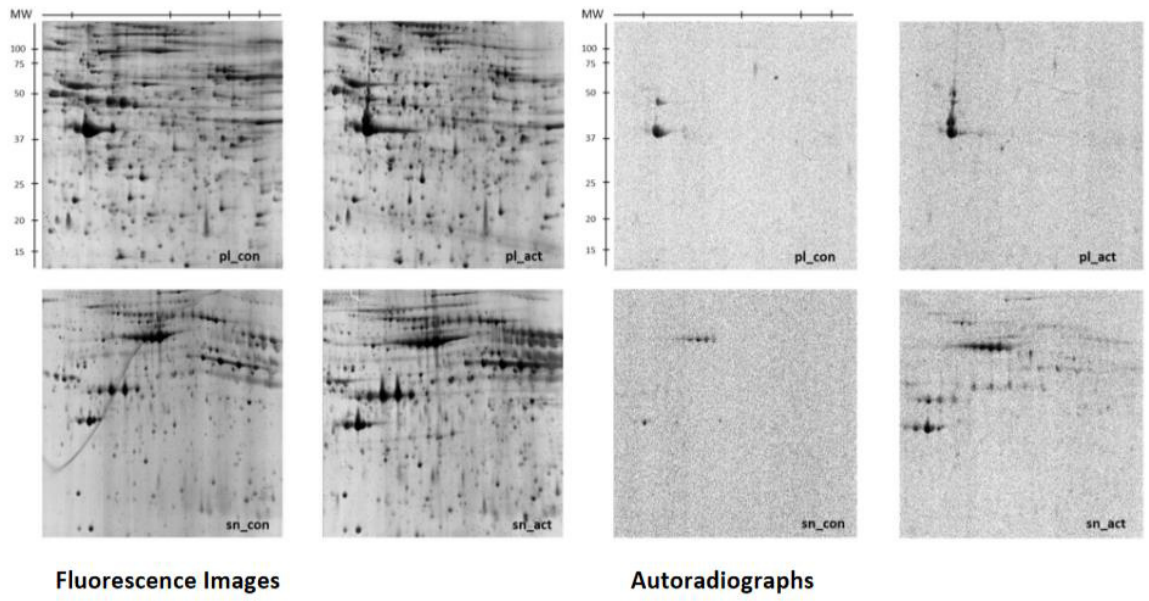


*Figure 1:* ***Fluorescence images and autoradiographs showing the synthesis of proteins.*** *Treated platelets (activated with ionomycin for 2 h)and control samples were cultured in $^{35}$S-methionine/cysteine medium. Platelet lysates (pl) and the supernatants (sn) were subjected to 2D-PAGE and the gels stained with RuBPS, and fluorescence images as well as autoradiographs were taken. Newly synthesized proteins were present especially in the secretomes of activated platelets.*

Platelets are anucleate cells in our bloodstream and are our first line of defence against haemorrhages. As they play an important role, blood clotting needs to be a highly regulated system as spontaneous aggregation of platelets can lead to arterial thrombosis and strokes.

As they establish their roles in hemostasis, vascular wall repair and thrombosis, research links platelets also to inflammation, healing, infections and even cancer.[1]The range of biological mechanisms that platelets seem to be a part of is steadily increasing. Even the invasion of pathogens, like parasites, bacteria and viruses seems to be recognized by the blood cells and lead to reactions through the release of signalling molecules and even changing of their shape. As platelets seem to react to highly recent or/and important developments like cancer, COVID and obesity, it seems essential to research their behaviour and investigate their potential for biomarkers.[2]

The research of platelets differs from other cells as their equilibrium is very fragile. They are so highly sensitive, it is an indispensable necessity to create a system to analyse the cells in their normal, inactivated state, as even the slightest change in temperature, brightness or chemical and biological surrounding can lead to their activation. Even a slight disturbance and activation may lead to affected cells, disturbing the control samples and therefore generating not representative data. Hence, one of the goals of this work was to develop a method to extract platelets and keep them as much at rest as possible, establishing the ground work for control samples. This was named the neutral resting point (NRP).
Any difference in expression could then be linked to the intended activation, generating outcomes and data, which can be used for further research.
To construct a representative system, it was decided to measure lysate and secretome of the collected platelets, so it would be comprehensible what molecules are already active, become active or inactive and get released by the cells.

To gather an overview over the mechanisms the established analytical method of proteomics was chosen, as well as a branch of metabolomics, lipidomics. As lipidomics is not yet as ingrained in the analytical methods, and lipids cannot be analysed universally, eicosanoids as a subgroup were chosen. Eicosanoids serve as fast acting signalling molecules between platelets and their surroundings.[3]
A method for extracting eicosanoids needed to be refined and their identification and quantification established.

Consequently, this work will try and work on those three scientific issues.

Establish the neutral reference point for platelets, activate them and review if they are able to synthesize proteins and develop a method to analyse eicosanoids in the secretome of said platelets.

# 2. Platelets, Biomarkers and Mass spectrometry

## 2.1. Platelets

Despite being the smallest blood cells in our blood stream, platelets display an enormous array of functions. Only discovered in 1882 by Giulio Bizzozero, these cells should be studied for a long time. Platelets are anucleate and disc-shaped blood components. Their main association is to hemostasis or the coagulation of blood. This system is to be regulated very carefully, since activation is only necessary when a blood vessel is damaged. Not only blood hemostasis is correlated to platelets, also the sensitivity to different diseases and inflammation leads to platelets being a good biomarker in blood.

The 2-3 µm platelets are formed by megakaryocytes in the bone marrow. Humans produce around 10^11 of them per day, while two thirds of them circulate in the blood, one third inhabits the spleen. They are dispelled by phagocytosis in spleen and liver.

Special to their structure, platelets do not have nuclei, but distinct mitochondria. Their membrane is a phospholipid layer and site of expression of several surface receptors and lipid rafts, which are required for signalling and intracellular trafficking. The specialized cytoskeleton helps the platelets to withstand the shear forces in the blood stream and maintain their discoid shape.

Platelets are involved in numerous functions like inflammation, tumour biology, host defence, maintenance of vascular tone, wound healing hemostasis, thrombosis and more.[4]

### 2.1.1. Platelet involvement in diseases

When damage to the vascular wall or the surrounding tissues occurs, platelets are the first blood particles to respond. Stimulations of platelets lead to their activation, release of adhesive proteins and of cytokines and mediators. They also can bind pathogens and release microbicidal proteins.

As platelets come in contact with other blood constituents, they influence lymphocytes, monocytes, granulocytes and endothelial cells. They assist white blood cells in migration and modulate inflammation, with releasing vascular permeability-enhancing factors, platelet

microbicidal proteins and chemokines, signalling molecules which lead to directional movement of different cell types.

Humans have around 150 – 400x10^9 platelets per litre of blood. This number is regulated by the number of megakaryocytes, the hormone thrombopoietin (TPO), and several immunomodulatory peptides. In case of infectious and inflammatory diseases the equilibrium between TPO in plasma and the rate of production of platelets is often disturbed. Especially acute viral infections lead to a decrease in platelet numbers and increased turnover. This phenomenon is also recognized with the introduction of bacterial lipopolysaccharide or tumour necrosis factors (TNF-α).

Thrombocytosis, the overabundance of platelets, is often observed in patients with malignant diseases and chronic inflammation. The hormone TPO is can be elevated in cases of cancer, autoimmune diseases like inflammatory bowel disease and septicaemia.[5]

The importance of platelet physiology in regards to vascular diseases is observed in the widespread use of aspirin, a cyclooxygenase inhibitor. The drug helps in the preventing of cardiovascular events, as well as acting as antithrombotic molecules in stroke and other prothrombotic conditions. It was also shown that low dose aspirin may reduce the spreading of present cancers and developing others, such as adenocarcinoma of the gut, and certain cancers of the lung, breasts and prostate. Precise mechanisms are not yet clear, but platelets are implicated in multiple stages of cancer progression.[6],[7]

Especially interesting for this work is their potential to generate molecules and not only depend on releasing prestored mediators. The synthesis of various proteins, nitric oxide, reactive oxygen species and lipids, like eicosanoids, which help with acute inflammation and hemostasis, shows the diversity of platelets.

### 2.1.2 Activation of platelets

One of the main functions of platelets is hemostasis and the associated blood coagulation. As a vascular injury is occurring sub endothelial collagen gets exposed and plasma von Willebrand factor (vWF) anchor into it. The interaction with the respective receptors on the platelet surface (Platelet vWF receptor – glycoprotein Ibα) leads to the activation of the platelets.[8] The binding of ligands to the GP receptors trigger the release of granule contents and change in the shape of the platelet. As the intercellular $Ca^{2+}$concentration increases platelets start to form pseudopods and the platelet fibrino receptors (GPIIb/IIa) are exposed. This leads to the formation of platelet plugs, which is called primary aggregation and is reversible. In the secondary activation the arachidonic acid thromboxane pathway and the ADP specific receptors, which are coupled with inhibitory G-proteins, mediate ADP-induced release of $Ca^{2+}$and activating the GPIIb/IIIa receptor which leads to further platelet aggregation and granulate release (as shown in Figure 2).[4]

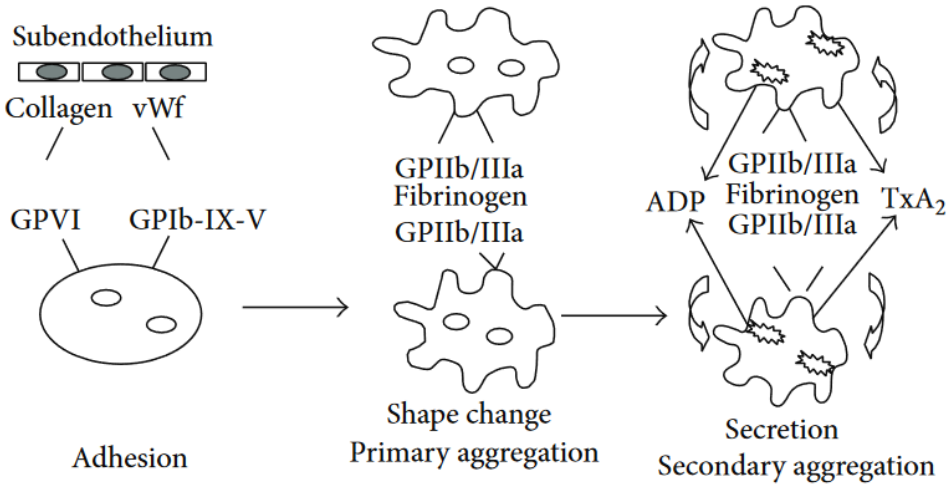


*Figure 2:* ***Activation of platelets after an injury in the vessel wall***
*Injury leads to the adhesion of platelets to the subendothelium surfaces and leads to interaction between receptors and the release of granule contents accelerating aggregation.*[4]

As the newly produced proteins and eicosanoids are the main focus of this work an activation of the platelets is needed. The mentioned rise of the cytosolic calcium levels can also be achieved by incubating the blood platelets with a $Ca^{2+}$-ionophore like ionomycin, without the need to activate the receptors on the surface. This receptor-mechanism independent

mechanism is a way to analyse the proteins of the releasate and the eicosanoids produced by the arachidonic acid thromboxane pathway. [9]

## 2.2 Biomarkers

Biomarkers, or biological markers, are by definition objectively evaluated and measured characteristics, be it in normal or pathological biological processes to an intervention. They help in the connecting of events with empirical results on a molecular level. Biomarkers can be used as early as in vitro studies, establish “proof of concept” experiments, help in safety evaluations and clinical studies. They provide a base for the designing of experiments and clinical trials by helping in the selection of molecules, their pharmacology and pathways.
Biomarkers can also be substituted for clinical responses, as they predict benefits and risks respectively. A widely known example would be the arterial blood pressure, reflecting the chance of stroke, cardiovascular diseases and death. It is to note that one biomarker alone can only display a certain spectrum of information.[10]

## 2.3 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique. Analytes, which can be solid, liquidous or gaseous are ionized and separated by their mass to charge ratio (m/z). This can be achieved by various ionization methods, e.g. electron beams. The mass spectra show the abundance of a certain ion in dependence of the m/z ratio.
MS can help to describe the structure of molecules and their mass, qualitative and quantitative composition of organic and inorganic samples, isotopic signatures and even examine the structure of surfaces. Mass spectrometers are one of the most complex devices in analytical chemistry, but all of them follow a more or less complicated set up. First there is a sample inlet, followed by the ion source, mass analyser and detector. To minimize the collisions with unwanted ions it is important to work under high vacuum.[11] As this work deals with extremely low concentrations there is a need to utilize a high-resolution mass spectrometer.

### 2.3.1 MS methods for biomolecules

MS has established itself as a core tool for large-scale analysis of biological molecules. In the near past there have been large advances in terms of resolution, sensitivity, scan rate and mass accuracy analysing proteins, lipids and metabolites. As genome sequencing became more established, the focus shifted to proteomics, a discipline to identify and quantify all the proteins of the proteome. To help with this endeavour, several methods were developed, with bottom-up and top-down as their most known.[12]

### 2.3.2 Introduction methods

As the samples become more and more complex it is necessary to gather as much information as possible. For this purpose, it is effective to couple chromatographic and spectrometric separation methods.[12] Chromatography dispenses the introduction of the analytes over time, based on their chemical properties. The base of chromatographic separation is the interaction of the wanted molecules with a stationary and mobile phase, with the exception of capillary electrophoresis, which uses electrical fields.

The most used introduction methods are gas chromatography (GC) and liquid chromatography (LC). In GC systems the separating happens as the stationary phase, the column, is being flushed by a mobile phase, mostly an inert gas.

In LC, analytes react with an oftentimes chemically derivated stationary phase. The liquid mobile phase changes composition over time. As the concentration gradient changes bound analytes get released and transported to the MS. LC is better suited for larger, more polar, molecules, as they are harder to get in gaseous phase and are often thermally unstable.[13]

### 2.3.3 Ionisation methods

To be detected by the mass spectrometer analytes must be ionized and brought into the gas phase. Currently electrospray ionization (ESI) is the most used ionisation method for –omics approaches, as it ionizes large, polar, non-volatile molecules from a liquid sample at atmospheric pressure. Together with Matrix-Assisted Laser Desorption Ionization (MALDI) and

Atmospheric Pressure Chemical Ionization (APCI), ESI is referred to as a soft ionization method as the molecules ionize without breaking chemical bonds. Electron Impact (EI) ionization is a harsh ionization method, as the analytes are bombarded with a beam of electrons. The high energy leads to destruction of mainly larger and more polar biomolecules.[14]

### 2.3.4 Mass analyser

The separation of the ionized analytes can be based on different principals, although all use static or dynamic electric and magnetic fields. The different types can be divided into two large groups, scanning analysers (like quadrupole instruments), which convey different masses in succession and those, which allow simultaneous transmission of all analytes (like the TOF analyser).

The main factors to characterize mass analysers are transmission, mass accuracy, resolution, mass range limit and scan speed. Combination of different analysers allows minimizing limitations and combining advantages. Hybrid instruments also allow collecting specific ions/precursor ions in a first analyser to then decomposition it and obtain spectra of their fragments/product ions.[15]

### 2.3.5 Analysis of acquired data

The raw data produced by the mass spectrometer needs to be processed further to achieve any knowledge gain. Noise reduction and feature detection are important and helpful algorithms to identify peaks and therefore allow identification and qualification. There are different components of a MS output. A spectrum contains all points with a fixed retention time (RT), the sum of all signals is a total ion spectrum (TIS). A fixed m/z range over all RT is called an extracted ion chromatogram (XIC).

After identification of the favoured features statistical analysis is indispensable.[13]

## 3. Proteins and Proteomics

### 3.1. Protein classification and structure

Proteins are biomolecules, consisting of chains of alpha-amino acids. They are macro molecules with a relative mass of 6000 to 1 000 000 Dalton.[16] Proteins are to find in all living organisms and are involved in all essential chemical processes. They can be organ-specific or species-specific.[17] α-amino acids are carboxylic acids, which have an amino functional group on the α-C-atom. Except for Glycin (R=H), all amino acids have an aliphatic, aromatic or heterocyclic group, which is specific for them and are chiral (Figure 3).

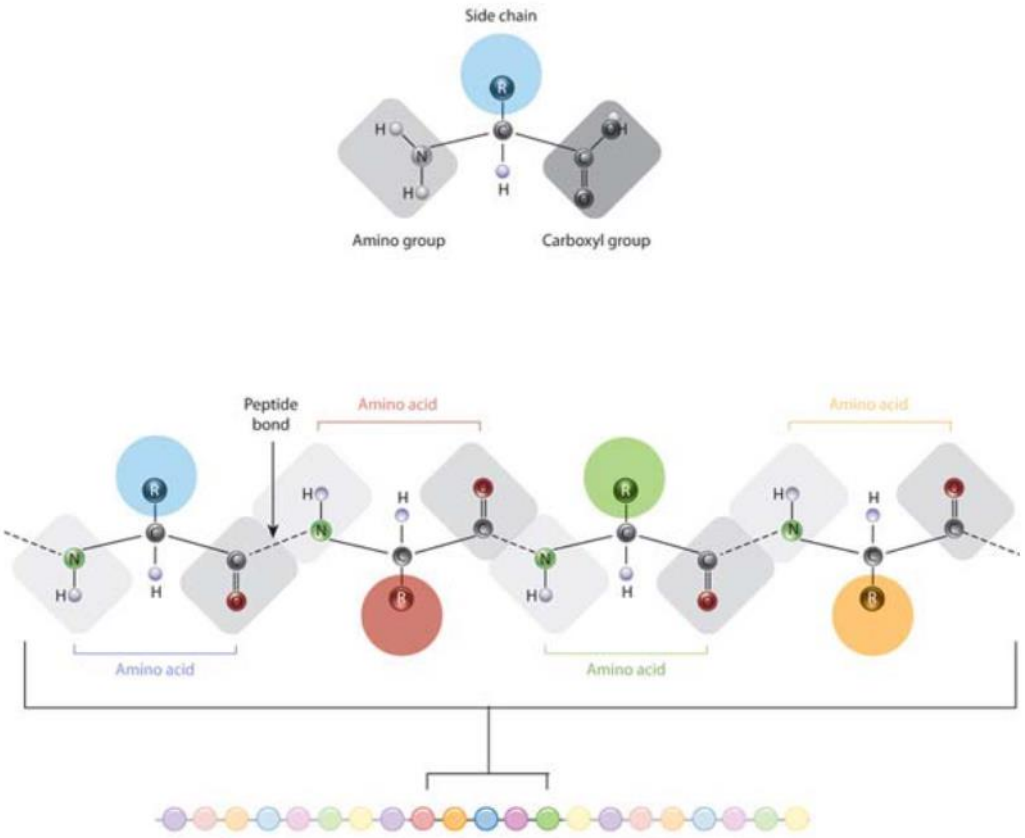


*Figure 3:* ***The linkage from amino acids resulting in peptides and proteins***
*The defining side chains of amino acids are pictured as colourful baubles. The connected amino acids form peptides and in larger number proteins.*[18]

In nature there are around 200 amino acids, while around twenty two are proteinogenic, meaning their combination leads to all the different proteins.[19] As proteins are linear chains of amino acids the sequence of them leads to the identity of the protein, giving them their structure and properties. The sequence is called the primary structure, which determines the

conformation of the molecule, which encapsulates the secondary and tertiary structure. The quaternary structure forms when there is an aggregation of different protein molecules.[20] The sequence of the amino acids is encoded by genes in DNA/RNA.

Proteins are involved in a wide array of functions. They span from very potent molecules such as hormones and enzymes to highly abundant ones as haemoglobin, carrier of oxygen in the blood and structural proteins necessary to give form to life.[27]

Since proteins are so abundant and deeply integrated in all bodily functions, they are destined to be used as biomarkers. [21]

## 3.2. Proteomics

Proteomics is the global analysis of proteins and therefore a highly dynamic field which is subject of change and improvement over time. Recent studies suggest that around 10 000 different proteins can be detected in human cell lines. As they cover a dynamic range of up to 9 magnitudes, analysis is everything but easy.[22]

### 3.2.1. History

One of the earliest tools of proteomics was developed in 1969, the two-dimensional gel electrophoresis (2D-GE). Proteins are separated by their isoelectric point (pI, the pH where the protein is uncharged) and size while migrating through a SDS-PAGE gel. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was combined with subsequent MS to identify polypeptides. The gel spots are cut out and the proteins enzymatically digested in gel. The peptide mixture is then analysed by MS or MS/MS. Low-abundance and hydrophobic proteins, as well as molecules with extremes in molecular mass and pI, are difficult to investigate with this method.

Now, the two main approaches are MALDI-TOF-MS and LC-ESI-MS/MS, providing higher sensitivity, specificity as well as speed and high throughput.

### 3.2.2. Sample preparation

With the potential of MS to detect the smallest amount of analyte, complexity and purity of samples are essential. With ion suppression (many different molecules compete for the charges throughout the ionization process) it is necessary to keep contaminations to a minimum. Examples include proteins from cell culture mediums, proteins coming from cell culture models or human and sheep keratin from skin, hair and clothes. It is therefore very important to include the possible contaminants in the searched data bases and use gloves and laminar flow hoods, wherever and whenever possible.[23] Platelets can be isolated from leukocyte-depleted platelet concentrates or freshly drawn blood, where the latter is to be preferred as it displays in vivo situations more closely. The contamination of other blood constituents has to be minimized, especially as the number of magnitude of concentrations can easily be overwhelming for the methods of measurement. As often equilibrium of quality and feasibility has to be found.[22]

### 3.2.3. Approaches to proteomics

MS-based proteomics for identification of proteins can be divided into different approaches.
Bottom-up proteomics cleave proteins in their peptides via proteolytic digestion for MS/MS analysis. Through comparing the mass spectra with in silico generated ones; peptides get matched and combined to possible proteins. As a special case of bottom-up, shotgun proteomics was established, where a complex mixture of proteins is digested and then analysed by high performance liquid chromatography (HPLC) hyphenated to a MS. This leads to enormous amounts of data which requires automatic data processing.
Top-down proteomics isolates an intact protein, which can be fragmented inside the MS. This leads to certain limitations as peptides are more easily ionized, fractionated and fragmented. To combine the strengths of both methods middle-down proteomics was introduced. In this strategy larger peptide fragments are analysed, minimizing peptide redundancy in proteins and helping retaining post-translational modification.[12]

### 3.2.4. HPLC and ionisation methods

One of the breakthroughs of proteomics was the introduction of soft ionization methods. ESI und MALDI are techniques that facilitate the opportunity to create intact gas-phase ions from large biomolecules.

ESI forms ion by pushing the analytes in solution through a fine needle at high potential and atmospheric pressure. The electric potential between the needle and the opening of the MS is normally between 2 and 5 kV and is responsible for the charge accumulation on the droplet. The beads shrink due to evaporation and increase their charge density. This creates a Coulomb repulsion which finally leads to an explosion. After the Coulomb explosion the smaller drops shrink until the analytes desorb into the gas phase.

MALDI ionizes intact proteins. The analyte is mixed with a matrix which absorbs energy at the wavelength of a laser, vaporizes and ionizes the sample. MALDI is often used for mass fingerprinting, as patterns are identified for differences in varying conditions.

As sample separation with MALDI has to be offline, ESI-MS allows being online with HPLC. With the ability to analyse complex and low sample amounts nano-HPLC is the most widely used separation method in proteomics. Due to the complexity of shotgun proteomics especially, one-dimensional LC is often not sufficient. In the case of analysing whole cell lysates it is necessary to introduce an additional separating column. Often the first separation column is a strong cation exchange (SCX) chromatography is used, followed by a reversed-phase one, to further the segregation and remove salts.[24]

### 3.2.5. Orbitrap

At the moment, Fourier transform ion cyclotron resonance (FTICR) and Orbitrap MS are the highest performing mass spectrometers for biomolecules. In both, ion packets are injected into the analyser and confined in an orbit around a spindle shaped electrode. They rely on Fourier transformation to determine the frequency of the motion in a magnetic or electric field respectively, followed by a mass calibration. FTCIR has a much higher resolving power, but has a slower acquisition rate and a necessity for cooling. Due to its high mass resolution, accuracy and sensitivity LC-Orbitrap MS has been the most widely used –omics platform.[25]

### 3.2.6. Protein identification

To identify a protein in bottom-up proteomics the MS features are compared to a database of previously identified or predicted features. With tandem MS (MS/MS) the most widely used approach is database searching, where peptide fragmentation patterns are compared to theoretical patterns in a database.

Another approach would be de-novo peptide sequencing or the combination of the two. For a given amino acid sequence possible masses and fragmentation patterns can be estimated. De-novo sequencing compares this data to the spectral patterns of the analytes and finds the most likely peptides. This needs no knowledge of the proteins beforehand and considers all amino acid sequences, which can be helpful in studying incomplete genome information.

In high resolution LC-MS it is possible to identify analytes by mass and retention time alone, or in addition use MS/MS fragmentation patterns, which also requires databases. Since tandem MS alone needs a lot of time and sample volume, hybrid approaches allow for higher throughput, as only a fraction of the analytes are fragmented again to obtain patterns.

In tandem MS/MS a precursor ion (most often the most abundant peak in a scan) is fragmented and scanned again. As the ion is fragmented with a neutral gas, collision-induced dissociation (CID) occurs and leads to a specific fragmentation pattern. CID leads predominantly to a breakage along the peptide backbone. The fracture of the amide bond leads to b- and y- ions, as shown in Figure 4. This specific fragmentation pattern functions as a fingerprint for a peptide and can be predicted and matched, leading to the identification of peptides and proteins.[26]

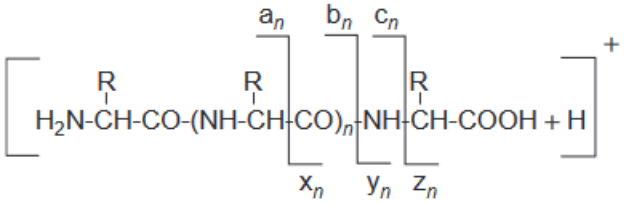


*Figure 4:* ***Peptide fragments derived from a double cleavage of the peptide chain by the means of CID***[15]

### 3.2.7 Platelet proteomics

The platelet proteome, the complete number of expressed proteins, include over 5 000 proteins and is vastly similar between healthy individuals. The main assignments of platelet proteomics are the measurements of quantitative changes in the amount of proteins, protein-protein interactions, localization and the study of post-translational modifications (PTM). It is from interest to gather inside of the composition of platelets and their compartments, as well their status of activation and health.

PTMs have a strong effect on the structure of proteins and can therefore change aspects such as function, localization, stability and more. MS has the ability to locate and identify and even screen for modifications. The most abundant PTMs is protein phosphorylation, which is regulated by protein kinases and phosphatases, allowing regulations of biochemical processes. Activation and inhibition of platelets are regulated by phosphorylation-dependant signalling cascades, changing the PTM patterns in milliseconds after activation. Besides phosphorylation, glycosylation and palmitoylation are also seen as important PTMs.[22]

# 4. Lipids and Lipidomics

## 4.1. Lipid classification and structure

Following genomics and proteomics, lipidomics again increases in complexity. Lipids have been defined, inter alia, as small hydrophobic or amphipathic molecules that originate by carbanion-based condensation of thioesters and/or by carbocation-based condensation of isoprene units. There is also a separation between simple and complex lipids, depending on their products on hydrolysis. It has been proposed to divide lipids into eight separate groups; fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides. Each of the categories also allows subdivision to enable even better definitions. As the classification is not based on a molecular structure; vitamins, hormones and certain components of cell walls can also fall under the umbrella term.
In this work the focus will be on fatty acyls (FA), on eicosanoids in particular.

## 4.2. Eicosanoids

FA are the major components of complex lipids. The group is characterized by a repeating series of methylene groups, which lead to their characteristic hydrophobic character.[27]
The term eicosanoids is used to describe a group of oxygenated, long chain polyunsaturated fatty acids containing 18 to 20 carbons. The major precursor of those molecules is the 20-carbon fatty acid arachidonic acid (AA), which lends the group its name (from Greek *eicosa* meaning twenty), pictured in Figure 5. The pathways leading to the oxygenated AA molecules are known as the arachidonate cascade. Other precursors would be related polyunsaturated fatty acids (PUFAs), like eicosapentaenoicacid (EPA) and linoleic acid (LA). With all eicosanoids it is very important to consider their stability in aqueous phases especially in regards of pH and time.

### 4.2.1 Groups of Eicosanoids

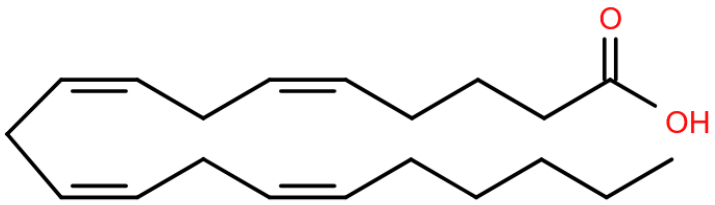


*Figure 5:* ***Arachidonic acid, the source of most eicosanoids*** *(structure taken from https://www.lipidmaps.org/databases/lmsd; accessed 12.08.2024)*

The three pathways are named after the necessary enzymes, cyclooxygenase (COX), lipoxygenase (LOX) and epoxygenase (EPOX). The first structures of eicosanoids were discovered in the 1950s and their pathways are studied since 1965.

The COX pathway leads to prostanoids, a class containing prostaglandins (PG) and thromboxanes (TX). For nomenclature; letters following the two-letter abbreviation and numerical and Greek subscripts denote numbers and location of bonds, side chains and substituents. Prostanoids are not stored in cells, but rapidly synthesized in a matter of seconds regarding extracellular stimuli. It transpires in 3 stages. The first is the mobilisation of AA from phospholipids in the cell membrane, then transforming AA to $PGH_2$ and lastly cell-specific conversion. In reaction to a stimulus, cell surface receptors release cytosolic $Ca^{2+}$ and activate more cellular lipases. For AA mobilisation, the most important lipase is the $Ca^{2+}$ dependant $cPLA_{2\alpha}$ (cytosolic phospholipase A2), which is directly involved, while Group IIA and Group V $sPLA_2$s work co-ordinately. Prostanoids act as local hormones, functioning near their site of origin. As an example, when platelets bind to subendothelial collagen, which gets exposed by injuring blood vessel walls, they synthesize $TXA_2$. The thromboxane promotes adherence and aggregation of other platelets and constricts the vascular smooth muscles.

Leukotrienes (LTs) are produced by the LOX pathway, by formation of a chemical reactive precursor leukotrien $A_4$ ($LTA_4$). As with prostanoids, LTs rely on the specific enzymes and are synthesized after cellular activation. As some of the most interesting eicosanoids derived from $LTA_4$, are $LTB_4$, a molecule with strong chemotactic and chemokinetik influence on leukocytes and $LTC_4$, which can constrict specific smooth muscles.

The last pathway EPOX is defined by the catalysation of various cytochrome P450 oxidases, which lead to three structurally distinct metabolite families, including Hydroxyeicosatetraenoic acids (HETEs) and Epoxyeicosatrienoic acids (EETs). Some metabolites from the EPOX P450 pathway show strong regulatory function on ion channels, membrane bound transport proteins and growth of mitochondria. [28]
Eicosanoids can also arise via non-enzymatic free radical mechanism, which will be not further discussed in this work.[29]

### 4.2.2. Eicosanoids in diseases

Although a lot about eicosanoids is not yet known, the activation of pro-inflammation cytokines accompanying infection seems to also release AA and related PUFAs, resulting in an eicosanoid storm. Analysing the data of all –omics disciplines it shows that eicosanoids can show pro-inflammatory and pro-resolution (anti-inflammatory) characteristics.
The production of eicosanoids and the expression of receptors are highly cell and tissue specific. However, the quantities of produced eicosanoids differ by different activation states or physiological conditions.
Studies of inflammation and eicosanoids have focused on the COX pathway and its products as they bring about known symptoms, like swelling, heat, pain and loss of function. Although it would be easy to associate the COX enzymes with pro-inflammatory products and group the metabolites of the LOX pathway with pro-resolution, it shows another dimension of complexity if both work together. Specialized pro-resolving mediators (SPMs) are produced by a combination of LOX and COX enzymes. SPMs are especially potent as they increase bacterial clearance and enable pro-resolution cytokine programs. Studies are even suggesting that information of pro-resolving responses could help in opposing antibiotic resistance. This shows the importance of focusing on the collective bioactivity, the affective cells and their proximity to each other.
Eicosanoids seem to play a role in a variety of diseases, even long studied and well-known ones as tuberculosis, Lyme disease and influenza virus infections. Drugs, that target the pathways producing eicosanoids have been used for centuries, e.g. aspirin, a Non-steroidal

anti-inflammatory drug (NSAID) hindering the PG synthesis through the inhibition of the COX enzymes.[30]

## 4.3. Sample preparation

As on the level of instrumental analysis, the main challenges in analysing lipids and in consequence eicosanoids are the low concentration, the grand concentration range of different analytes in one sample and the number of structurally similar compounds.
There is nearly always a similar approach; separation by reversed phase chromatography, ESI in negative mode, following detection on a highly sensitive triple quadrupole MS in selected reaction mode. This shows the importance of optimizing the surrounding processes.

As eicosanoids are so quickly regulated, sampling has to be as deeply under scrutiny as the more technical aspects of analysis.
As it is easier to obtain samples from cell culture; they lack the complexity that specimens from living donors can provide, although selecting representative individuals and determining the amount of sample needed can be challenges in it of themselves. As blood carries a large variety of different lipids and in an enormous range of concentrations a purification step is indispensable.
Ostermann at al. compare in an extensive work the differences of liquid-liquid extraction (LLE) and several solid phase extraction (SPE) methods concerning extraction efficiency from plasma, recovery and reduction of ion-suppressing matrix.[32]
Storage also needs to be planned in advance since aspects of biological samples can be highly sensitive to factors as time and temperature. Especially signalling molecules as eicosanoids are extremely endangered to degradation by enzymes, which can lead to changes in concentration and species in lipids.
To circumvent this problem analysation as quickly as possible is recommended or to decimate the proteins and extract the lipids. Should that not be possible, the samples should be stored at cold temperatures. The goal is to lower the temperature as quickly; and store as low as possible. Further problems could be the auto-oxidation which leads to a falsification of the results or the degradation of sample vessels due to the need of using organic solvents to keep the lipids in solution.[31]

As to ensure quality control isotope-labelled internal standards (IS) are an essential tool. They ensure the correctness of every step and need to be added as soon as possible. To ensure that standards are behaving as similar as possible the desired analytes is to use isotopes, which often contain $^{13}C$ or $^{2}H$ atoms. In the MS the isotopes are clearly differentiable, but the physical and chemical properties are the same. Problems arise if there are not enough internal standards available, as lipids show a lot of diversity. It is advised to use at least one standard per group of eicosanoids. Even though the IS is similar to the different analytes, a deviation in retention time occurs. Consequently, a difference in ion suppression is not shown by using an isotope labelled IS, which underpins the importance of removing coeluting matrix components.[32], [29]

## 4.4. Lipidomics

In accordance to the other –omics disciplines; the entire collection of chemical distinct lipids in a defined biological system is called the lipidome. Therefore, the identifying of structures, the quantification of lipids for pathway analysis and search for biomarkers as well as the determination of interaction of lipids with biomolecules is summarized as lipidomics. As this represents such an extensive field, subcategories emerged to reflect their particular focus. Under the –omics umbrella, lipids are considered metabolites. However, as lipids show chemical uniqueness and are so specific in their function a separate branch was established. At first studies focused solely on one species or class of lipids or one pathway or single cell, which quickly led to the need to connect information on a grander scale. The rapid evolvement of analytical possibilities opened the door to view the metabolism of whole organelles, organs and systems.

The first mention of the word 'lipidome' was in 2001[33], while `functional lipidomics' was established only a year after as membrane lipids were the first to be of high interest.[34]

The number of lipids on a cellular level can be estimated to be in the millions. Consequently, the vast majority of lipidomic research is gathered from experiments measuring the differences in physiological or pathological states of systems.

### 4.4.1. Platelet lipidomics

As in all mammalian cells the most abundant lipids in platelets are phospholipids. They arrange themselves as membranes, where the hydrophobic fatty acids are oriented to the core and the more polar headgroups are facing the aqueous surrounding. Those membranes also contain signalling areas, names liquid rafts, encompassing sphingomyelins and free cholesterol. Even as early as 1962 a comprehensive analysis of platelet phospholipids was achieved by Marcus et al.[35], leading to various studies in the seventies and eighties comparing lipid compositions of platelets in different subject groups and species. Eicosanoids were first identified by Hamberg and Samuelsson[36] in 1974 in Stockholm, at the same time as Vane in London. Their work was rewarded in 1982 with a Nobel Prize. In the following years lipids were characterized and their attributes described, until the overall composition of resting and activated platelets was established.

### 4.4.2. Shotgun lipidomics

As already outlined LC-MS/MS is the most abundant used approach at analysis in the –omics fields. In lipidomics there is a different approach called shotgun lipidomics. While LC provides separation via chromatography, shotgun lipidomics uses a direct infusion of a complex lipid mixture into the MS. In the early nineties this direct infusion was used to avoid difficulties from variations in concentration, problems in the chromatography and ion pairing alterations. Advantages are a high throughput of samples, although it is not quantitative and low abundant species, which are frequently highly active and relevant, are often missed.[37]

### 4.4.3. Chromatography

With the development of lipidomics several chromatographic techniques were developed and refined. The main focus was to separate intact phospholipids, which disqualifies GC and GC-MS immediately as derivatization is necessary. Thin-layer chromatography (TLC), which was a convenient and popular tool, is now mostly replaced by HPLC, although there have been new approaches in coupling it with MALDI to provide precise structural information. Other

techniques in phospholipid separation are column chromatography and the subsequent solid phase extraction with prepacked cartridges.

The first application of HPLC with regards to phospholipids was in the 1970ies and showed advantages in throughput, resolution and sensitivity. Major benefits are also the option to work at lower temperatures and limiting the exposure to oxygen which reduces the risk of isomerization and auto-oxidation. The separation of lipids shows mainly two options of operation of HPLC in view of lipids. Normal phase HPLC has been mainly applied to separate the different classes of phospholipids, while reversed phase HPLC is predominantly used to isolate certain lipid species within the classes.

For the separation of eicosanoids RP-HPLC is used as the isolation of molecules should occur mainly on their hydrophobicity, meaning the length of the fatty acid chain, additional heteroatoms and the number, position and configuration of double bonds. The non-polar stationary phase provides interaction with the fatty acid chain rather than the polar head group and lead to a separation based on the necessary factors to differentiate eicosanoids.[38]

### 4.4.4. ESI

For either shotgun lipidomics or LC-MS coupled lipidomics ESI is the most used and approachable source of ionisation. The usage of organic solvents is especially useful for lipids, as they increase their solubility. To ease the ionization, compounds that increase conductivity can be added to the sample. ESI showing minimal in source fragmentation and therefore higher ion intensity is an important benefit in measuring low abundant species like eicosanoids. As the lipids enter the MS intact, mostly predetermined masses can be detected and lead to better identification quantification, as well as regulated fragmentation in the MS. Additionally, ESI-MS can be so delicate that dimers, complexes and solvent adducts can be measured. The addition and variation of using it in negative or positive mode, flow rates, solvents, modifiers, like acids, bases and salts, can lead to better separation in the chromatography as well as form special adducts and enhance therefore signals of wanted lipids. The ESI itself works therefore like a separation step, favouring lipid classes or individual species with higher efficiency and lower ion suppression.

The limit of detection is around amol/µL and the sensitivity for ESI-MS is improving constantly.[29]

### 4.4.5. Q Executive

Similar to proteomics, lipidomics requires a mass analyser with high mass accuracy and resolution. As metabolomics deals with even lower abundant molecules these factors need to be even more emphasized which leads to time of flight (TOF) and quadrupol/iontrap analysers. As ESI offers minimal in source fragmentation, it is possible to gather information in breaking the molecules in a controlled environment, leading to MS/MS. The ability to do so requires mass spectrometers to have multiple mass analysers or an iontrap. The Orbitrap system combines devices and was used for this works purposes (Figure 6).

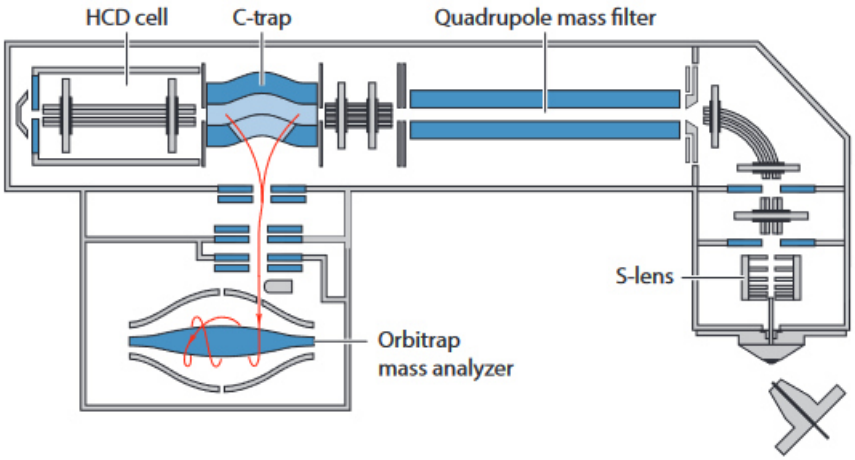


*Figure 6:* ***Schematic of the architecture and scan schema of the Orbitrap QExactive mass spectrometer.***[39]

From the ESI source the ionized analytes are led into the RF lens, where they are focused and directed into the first quadrupol analyser. Quadrupols are designed to stabilize ions through radio frequency. They can either stabilize paths of all ions or fixed on a select ion with a defined m/z ratio. The quadrupol directs the ions to the C-Trap, an iontrap which can collect ions and release them selected by m/z into either the Orbitrap analyser or the collision cell. In the collision cell the analyte ions collide with a chosen collision gas at a certain energy to induce fragmentation, which provides important information. The fragment ions get directed back to the C-Trap and further to the Orbitrap, which functions as the mass analyser.

### 4.4.6. Analysis of acquired data and bioinformatics

With every iteration of the –omics studies the complexities of generated data grows. As the structure of the metabolome is vastly diverse the generated data is not easily catalogued. Since lipidomics is a relatively recent field in comparison to genomics or even proteomics, the automation of data processing is not yet available.

Bioinformatics helps with the development of databases and statistics to help with management of large amounts of generated data of biological research.

The foundations for automated identification of lipids are databases that store information of known molecules to offer the opportunity for comparison. That information can include structure, exact masses, MS/MS spectra for different ionization modes, isotope patterns, different adducts and ion forms, retention times for LC and more. One of the largest databases which houses data of over 48 500 molecules at the moment is the LIPID MAPS Structure database (LMSD) hosted under https://www.lipidmaps.org/databases/lmsd. Often these databases are a constant work in progress and can be seen as a starting point. Software to process data at speed and facilitate demonstrative data is also necessary.[29]

### 4.4.7. MS/MS characterisation

To identify lipids from their mass spectrometry spectra leads to obstacles, due to their isobaric nature since a lot of lipids, especially eicosanoids, derive from the same base molecule/s. As the fundaments of a lot of eicosanoids are the same PUFAs, the differences are often minor like the positions of double bonds and hydroxyl groups.[40] The variability is so miniscule that the difference in velocity of the LC is sometimes minimal, leading to the necessity to finesse the HPLC measurements.

To gather more concise information lipidomics uses CID to ensure identification, but unfortunately other than proteins, the fragmentation patterns of lipids are not easily predictable. Since the early days of lipidomics it is shown that some lipids show specific patterns in fragmentation related to their structure and make matching easier.[41] Apart from all difficulties, it is possible and feasible to detect variations in fragmentation patterns and distinguish different isomers of eicosanoids. Databases are indispensable for this endeavour. The fragmentation patterns of the CID and the conditions of the measurement need to be

accessible. LipidBlast, hosted under https://fiehnlab.ucdavis.edu/projects/lipidblast, is an in-silico tandem MS database and software for lipid identification covering nearly 120 000 compounds from 26 lipid compound classes, based on data of other data bases.[42]

# 5. Materials and Methods

## 5.1. Preparation of blood platelets and activation with ionomycin

As established in the introduction the development of the neutral reference point, to find an isolation method which approximates platelets at rest, is a central part of this work.
It took four experiments to arrive at the following isolation protocol, which will be outlined accordingly.

Platelet isolation and activation

To collect the platelets, peripheral venous blood was drawn from four healthy donors with written consent of each donor and the approval of the Ethics Committee of the Universitätsklinikum Regensburg (ClinicalTrials.gov Identifier: NCT01614301; Prof. Dr. Albrecht Reichle).
To ensure that the platelets did not experience premature activation by too much contact with the needle puncturing the vein and get the least interference with factors on the outside of the bloodstream, the first few drops were discarded.
Two pre-opened tubes, containing 3,2% sodium citrate (Vacuette system; Greiner, Kremsmuenster, Austria) were used per donor. It was important to let the blood drop freely, without as much disturbance as possible. The tubes were closed and inverted slowly four times to mix throughout. To obtain platelet rich plasma (PRP) the samples were centrifuged for 20 minutes at 100g, which yielded around 1mL per tube.
To achieve further purification of the PRP to obtain samples with even fewer interfering substances a size exclusion chromatography was performed. Plastic columns were plugged with a ball of cotton wool and filled with 10mL Sepharose 2B (Sigma, Germany), which before and was mixed with 10mL RPMI medium (RPMI medium 1640 (1X) [+]-Glutamin, Gibco, UK), containing no FCS.
After equilibration of the columns, the 1mL of the PRP was added. For the elution RPMI medium was used. After an elution volume of 2,5mL, the next 1,5mL, which contained the platelets were collected in 15mL plastic tubes. Fractions, belonging to the same donors were pooled and kept in a temperature controlled and dark place for one hour. The tubes were

carefully turned periodically. Then the platelet concentration was measured using a MOXIcell counter (Orflo Technologies, USA).
The samples of the PRP were divided in three 1mL fractions, one used as the control; the other two were for platelet activation. This was performed by adding 100µL of 10 µM Ionomycin (Sigma, Germany) dissolved in PBS, to a final concentration of 1 µM, as 0,3 to 3 µM was recommended by literature.[9] The control samples were centrifuged at 1500g for 10 minutes. The activated samples were treated the same way after 15min and 3h after activation respectively.

Experiments

In the first experiment there were 4 donors. Instead of RPMI medium, phosphate buffered saline (PBS) (calcium-free Dubecco's PBS, GIBCO, Paisley, Scotland, UK) was used. The platelets also rested for only 10 minutes and the activation was conducted consecutively. There was also only one control and one activated sample with an activations time of 10 minutes. This was a proof of concept trial and just established the possibility of gaining data.

The second test used RPMI medium as the platelets of the control group showed indications of activation and the resting period was extended to one hour to ensure a mostly undisturbed environment. The activated samples were increased and set at 15 minutes and 3 hours.

After the third experiment the concentration of various chemicals was set and the concentration of platelets was measured, with MicroDiff 18 Blood Analyser (Coulter Electronics) at 1 to 3x10^6 cells/mL.

One major difference to the current SOP up until this point was the use of a C18 column (BioRad, Hercules, 15mm diameter) instead of a plastic column plugged with cotton, which led to blockage, with the need to use vacuum and fewer platelets in the resulting sample.
As the fourth test was out of state, without the normally used lab equipment and under time restriction, it forced the preparation of all samples and the following activation to happen at the same time. This led to a decrease in the variation of the biological samples and higher time efficiency.

The final experiment, implemented all the advancements and lead to a concentration of 1,8 to 2,5x10^7 cells/mL.

Photos were taken at 40 x magnification with the Zen 2011 (blue edition) Version 6.1.7601 by ZEISS to compare the resting platelets and it shows that the platelets were round and not deformed (as shown in Figure 7).

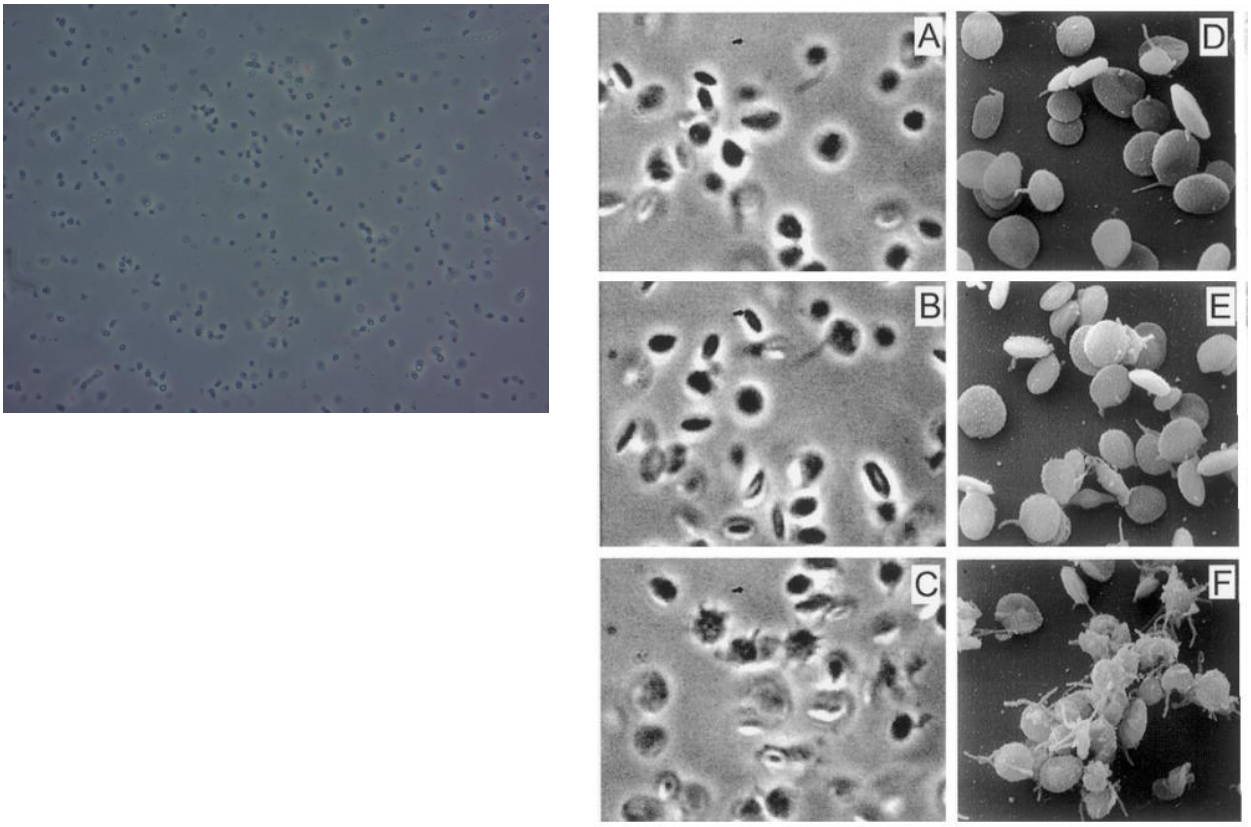


*Figure 7:* ***Platelet shapes***
*The left picture shows the control group of platelets at rest at 40x magnification, by the author. The right pictures were taken from the paper 'Room Temperature Activates Human Blood Platelets' by Spurejet al.. They show aggregation induced by only temperature changes. A-C show phase contrast micrographs of live platelets at 35 000 magnification. D-F are transmission electron micrographs. A and D are at 42 degree Celsius, B and E at 37 degree Celsius, C and F at 20 degree Celsius, illustrating the sensitivity of platelets to their surroundings.*[43]

## 5.2. Proteomics

The proteomics part of the experiments after the obtaining of the samples is already long established in the group of Professor Gerner. Nothing was changed in terms of concentration, digestion or measurements of the proteins or peptides respectively.

### 5.2.1. Protein extraction of platelet lysates and secretomes

The previously acquired cell pellets were covered with 100µL sample buffer (7,5 M urea, 1,5 M Thiourea, 4% CHAPS, 0,05% SDS, 100 mM Dithiothreitol) and put on ice. To obtain proteins of the whole cell lysate the pellets were lysed by sonification with an ultrasonic homogenizer. After centrifugation at 1500g for 5 minutes, a few crystals of urea were added and kept at -20 degree Celsius. The other samples were put through the exact same procedure 15 minutes and 3 hours respectively after the adding of the ionomycin.

The supernatants were transferred in a new tube holding 4 mL of ice-cold absolute ethanol (Merck, Germany), mixed and incubated at -20 degree Celsius overnight (or at least for 18h) to precipitate the proteins of the secretome. The next day the samples were centrifuged for 20 minutes at 4750g and 4 degree Celsius, the resulting supernatant was used for the lipidomics analyses. The protein pellet was dried upside down in a desiccator for 10 minutes or until dry. 50µL sample buffer and a few crystals of urea were added and incubated over night at -20 degree Celsius.

The next day all the protein samples were thawed, vortexed, centrifuged at 1500 rpm for 5 minutes and then put in an ultrasonic bath. The samples were transferred into new vials and sample buffer was added to obtain 100µL.

### 5.2.2. In solution digest

After the determination of the concentration of proteins in all samples via the Bradford assay[44], concentration and filter-aided digestion, after the original described protocol[45] was performed. 20µg of each protein sample was concentrated onto a washed 10kD MWCO filter (Pall Austria Filter GmbH). Dithiothreitol (DTT) was added to reduce the proteins and

incubated for 30 min at 37 degree Celsius. After washing, iodoacetamide (IAA) was introduced for alkylation and again incubated for 30 min at 30 degrees in the dark. Proteins were washed with cold 50mM ammonium bicarbonate buffer and then 10µL of trypsin/LysC mix (Promega, Madison, US;0.1µg/µL) for digestion was added before mixing on a shaker at 1100rpm for 10 min at 4 degrees. The samples were incubated for 18h at 37 degree Celsius. Another 5µLfresh trypsin/LysC mix was added and the digestion was completed for further 6h at 37 degree Celsius. Thereafter, peptides were eluted with 50µL MS-grade water and subsequently, to guarantee to gather also the hydrophobic peptides, 50µL 0.5% trifluoroacetic acid (TFA). The finished samples were dried via vacuum centrifugation at 40 degrees for 1 to 2 hours and stored at -20 degree Celsius prior to LC/MS analyses.

### 5.2.3. Analysis of the samples using HPLC-MS/MS

#### 5.2.3.1. LC-MS method

Samples were brought into solution in 5µL 30% FA containing 10fmol each of 4 synthetic standard peptides and diluted with 40µL mobile phase A ($H_2O$: ACN: FA = 98: 2: 0.1). Of this solution, 10µLwere injected into the nano HPLC-system (Dionex Ultimate 3 000) and peptides were loaded on a 2cmx75µm C18 Pepmap100 pre-column (Thermo Fisher Scientific) at a flow rate of 10µL/min using mobile phase A. Peptides were then eluted to a 50cmx75µm Pepmap100 analytical column (Thermo Fisher Scientific) with a flow rate of 300nL/min, using a gradient over 43min beginning with 7% and ending up with 40% mobile phase B (ACN: $H_2O$: FA = 80: 20: 0.1) for secreted proteins, and a gradient over 235min beginning with 8% and ending up with 40% mobile phase B for proteins from platelet lysates. The nano HPLC system was coupled to a QExactive Orbitrap with a nanospray ion source (Thermo Fisher Scientific), where scans were performed in the range from m/z 400 to 1400 at a resolution of 70 000 (at m/z =200), MS/MS scans at a resolution of 17 500 (at m/z =200), using a top 8 method and applying HCD fragmentation at 30% normalized collision energy.

### 5.2.3.2. Identification and label-free quantification of proteins

Identification of proteins and label-free quantification (LFQ) as well as statistical analyses were performed using the MaxQuant 1.5.2.8 software including the Andromeda search engine and the Perseus statistical analysis package. Proteins were identified using the UniProt database for human proteins (version 102014 with 20 195 entries, restricted to reviewed entries only), a peptide mass tolerance of 25ppm, an MS/MS match tolerance of 20ppm and a maximum of 2 missed cleavages. Carbamidomethylation of cysteines was further set as fixed modification, methionine oxidation as well as N-terminal protein acetylation as variable modifications. A minimum of one unique peptide was required for positive protein identification. Match between runs was performed with a 5min match time window and a 15min alignment time window. For peptides and proteins, a false discovery rate (FDR) of less than 0.01 was applied. As MaxQuant-derived data are not yet supported for complete submissions, the Proteome Discoverer 1.4 running Mascot 2.5 and Uniprot for human proteins (version 112015 with 20 193 entries, restricted to reviewed entries only) was used as search engine. Actually, all proteins found to be regulated via MaxQuant were positively identified by Proteome Discoverer as well.

### 5.2.3.3 Determination of protein regulation

Protein regulation was determined by comparing the LFQ values for each individual protein in platelets at different functional states, using the Perseus statistical analysis package. The same initial protein amount of 20µg used in all experiments served for normalization. Differences of protein abundance values before and after activation of platelets were determined by a two-sided t test. Two-fold changes with $p<0.05$ were taken as a minimum to consider proteins as significantly regulated.

## 5.3. Lipidomics

Lipidomics was constantly refined as part of this work and was subject of continuous improvement.

### 5.3.1. Extraction of eicosanoids via solid-phase extraction

The supernatants which were kept after the centrifugation of the secretome proteins were processed as follows. To the samples containing the lipid fraction three internal standards, (PGE2-d4, 15S-HETE-d8 and PGF2a-d4; Cayman Europe, Tallinn, Estonia) were added to reach a final concentration of each of 100nM (Table 1). The ethanol was evaporated via vacuum centrifugation at 37degree Celsius for about 40 min until the original volume of sample - before protein precipitation, around 1 mL - was restored. Samples were diluted 1:3 with MS-grade water to ensure a good compatibility with the SPE, vortexed and ultra-sonicated for 5min in a water bath at room temperature.

Eicosanoids were then extracted using 30mg/mL StrataX solid phase extraction columns (Phenomenex, Torrance, CA, USA). Over the whole process the column was never dry and the flow of the liquid was kept at a steady 1 drop per 2 seconds. Columns were conditioned and equilibrated with 2mL HiPerSolv grade methanol (MeOH, VWR International) (VWR International, Vienna, Austria) and 2mL LC-MS grade water (Sigma-Aldrich). After sample loading, the columns were washed with 15% MeOH, and eicosanoids were then eluted with 1mL (twice with 500µL) with a mixture of acetonitrile (ACN, VWR International): MeOH: formic acid (FA, Sigma-Aldrich) (49:49:2) into a glass vial. Solvents were evaporated via vacuum centrifugation at 37 degree Celsius and lipids were dissolved in 200µL reconstitution buffer (ACN: $H_2O$: FA, 30:70:0.02).

| Synonym | m/z | Systematic Name |
|---|---|---|
| **5S-HETE-d8** | 327.278 | 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5,6,8,9,11,12,14,15-d8) |
| **PGF2α-d4** | 357.2585 | 9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid (3,3,4,4-d4) |
| **PGE2-d4** | 355.2428 | 11R,15S-dihydroxy-9-oxo-5Z,13E-prostadienoic acid (3,3,4,4-d4) |

*Table 1:* ***The name and m/z of the used deuterated eicosanoid internal standards***

Experiments

In this particular step, the following determining factors were investigated and optimized.

The washing step after the loading of the sample was slightly changed. Before the column was washed twice with MS-grad water, which was changed to 15% MeOH in water, as pure water lead to a decrease in measured eicosanoids.

The elution volume was as well changed by an increase of 100% from eluting twice with 250µL, to twice of 500µL.

Also, the vacuum centrifugation via SpeedVac (Thermo Scientific) was under scrutiny. Evaporation via $N_2$flow was also tested, but did not lead to any improvement.

### 5.3.2. Analysis of the samples using HPLC-MS/MS

#### 5.3.2.1. LC-MS method

Samples were pipetted into 200µL glass inserts and capped with Teflon caps. All eluents (mobile phase A (H2O: FA, 100:0.02) and mobile phase B (ACN: MeOH: FA, 90:10:0.02)) and solutions were degassed prior to their usage. For the chromatography a Vanquish (Thermo Fisher Scientific) UHPLC System was used. A 40min gradient flow method was established using a Kinetex™ 2.1mm x 15cm, 2.6 µm, C18, 100Å reversed phase column (Phenomenex). The flow rate was set to 250µL/min and a gradient was applied (0-2min: 5% mobile phase B, 2-28min: 5-85% mobile phase B, 28-35min: 95% mobile phase B and finally re-equilibration

with 5% mobile phase B until next run). 20µL of the samples were injected and analysed in technical triplicates. After each sample, the whole method ran with a blank to ensure the minimization of carry over and overload of the column. The samples were not frozen or stored for a long time after the SPE, but cooled at 4 degrees Celsius and/or kept at the same temperature in the autosampler of the UHPLC.

#### 5.2.2.2. MS method

To analyse the samples were eluted into the HESI source of a QExactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) to achieve negative ion mode ionization.

The first quadrupole was set to select ions with m/z between 250 to 750 and a resolution of 35 000 (at m/z = 300). At first the MS performed a full scan and generate MS1 spectra. A lock mass of 344.9791m/z was set**;** drift was +/- 5ppm over all experiments.

Then MS/MS scans of the 6 most abundant were produced by sending them to the collision cell and perform HCD fragmentation at 30% normalized collision energy and analysed in the Orbitrap at a resolution of 17 500 (at m/z = 300).

For further improvement of the analysis by targeting the analysis of lower abundant peaks; a dynamic exclusion list was established. This list included previously obtained m/z values which then were excluded from being selected for the next four seconds.

#### 5.2.2.3. Identification and quantification of eicosanoids

The identification of eicosanoids presents some specific challenges, which need not only a bioinformatic approach but utilize manual steps as well. The raw files, which were generated by the Orbitrap were analysed and scored manually using ThermoXcalibur 2.2 Sp1.48 (Qual Browser).

The measured lipids were searched against the lipid mass structure database (LIPID MAPS Lipidomics Gateway, http://www.lipidmaps.org/) and used as references.[46],[47]

At first it was determined which eicosanoids were present in the samples by establishing certain standards. At the base step the exact mass had to be identical, which was searched

against the database using a group made software. After checking for masses correlating to biological active eicosanoids the next step was to compare the pattern resulting from MS/MS fragmentation. Here the most important information includes the recorded masses and their ratios to each other. It was agreed to compare the three most abundant fragment peaks and their relative intensities with the measured data (Figure 8). It has to be noted that the information in the database was generated by a multitude of varying labs and groups, as well as different HPLCs and MS with different settings. This detail needs to be considered as ratios of the fragmented molecules and therefore their patterns can differ.

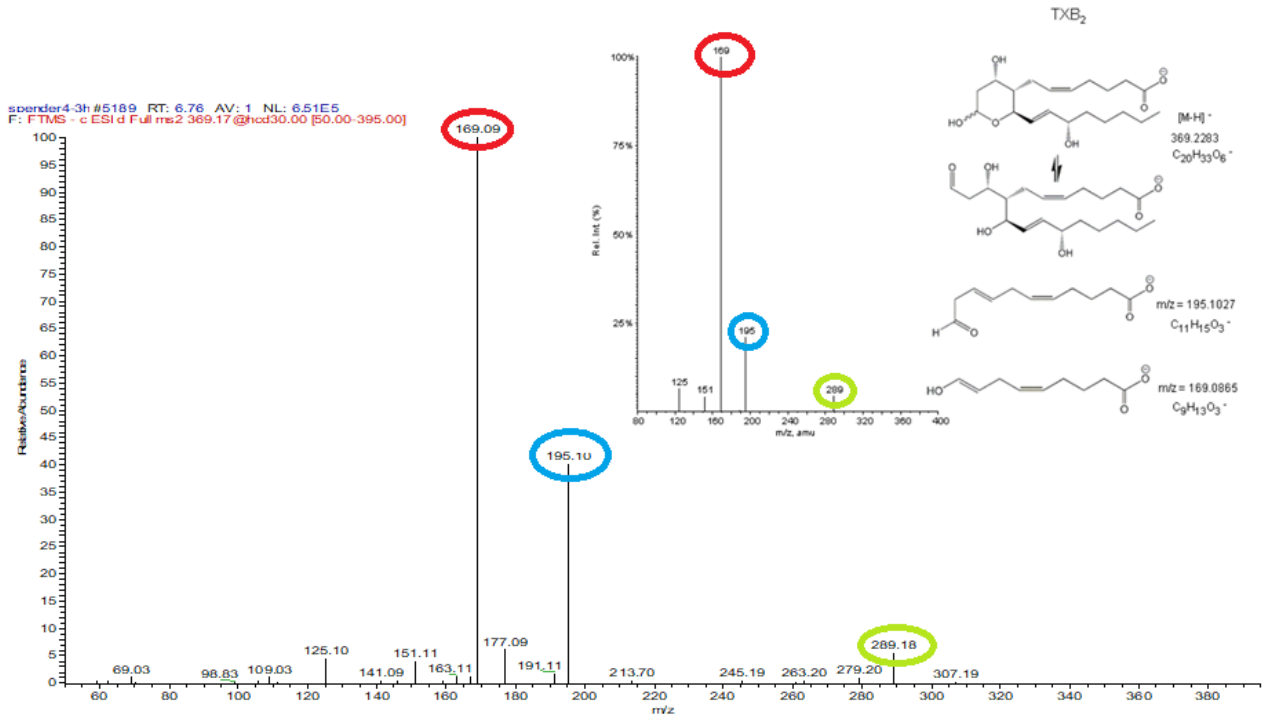


*Figure 8:* ***MS/MS fragmentation patterns $TXB_2$***

*The left depicts a MS/MS spectrum of $TXB_2$from the sample of patient 4 after 3 hours of incubation time after activation. Precursor mass of 369,17 and RT of 6,7 min was chosen. The right shows the fragmentation pattern which was taken from the lipid maps structure database. Highlighted are the three fragments with the highest intensities. Both spectra show the same fragments and patterns and ensure therefore a positive identification.*

Since the platelets present a reactive system, not all lipids were found in all of the samples, due to their lower concentrations especially not in the control samples as the cells where at rest. Even if a peak was found, not all could produce a MS/MS pattern. In this case exact mass and retention time, in correlation to the fitting IS was used to identify peaks and their equivalent eicosanoid. The conditions were set that the retention time had to be in a window of three times the peak width of the analysing peak in correlation of that to the IS and a fragmentation pattern had to be present in at least one sample.

After the identification of the eicosanoids, the corresponding peaks were searched across all samples and the area under the curve was determined. For the quantification the areas of the internal standards were assessed and used for the internal calibration and also normalized over the number of platelets.

# 6. Results

The three main tasks of this work were to establish the NRP, review if platelets are able to synthesize proteins and examine their expression of eicosanoids. To verify those claims it is needed to interpret the following results.

## 6.1. Blood collection and activation

Blood was collected in the most cautious and careful way, letting it drop freely and discarding the first drops to avoid sheer stress, contamination and therefore activation of platelets. A major point would be that no further anticoagulant like prostaglandin E1 was necessary. Only sodium citrate to bind any $Ca^{2+}$ was used.

Activation of the platelets was induced by using the calcium ionophore ionomycin, which induces the ions mobilisation. Apart from that, no premature, unwanted aggregation was observed, as platelets did not release more lipids before the planned activation and the samples would not show the uniform trends that were observed, as well as the following indications.

## 6.2. Proteins

### 6.2.1. Profiling of lysates and secretomes of control and treated platelets

Through the help of database search algorithms 2 543 protein groups (with a FDR[48]of less than 0,01) were determined at both peptide and protein level. To assign those to biological processes, the data was submitted to the DAVID functional annotation tool[49]. Apart from hemostasis, as the main function of platelets, platelet proteins are involved in different processes such as protein localization, transport of proteins, including vesicle mediated transport systems, furthermore membrane and cytoskeleton organization, small GTPase mediated signal transduction, as well as proteasomal proteolytic processes, and finally generation of precursor metabolites and energy (as seen in Table 3A).

Upon activation with ionomycin protein regulation was observed and shown in Table 2.

| Location | Significant regulation | Number of regulated proteins 15 min/3 h after addition of ionomycin) |
|---|---|---|
| **Lysate** | ↓ | 243/317 |
| | ↑ | 30/32 |
| **Secretome** | ↓ | 32/111 |
| | ↑ | 184/269 |

*Table 2:* ***Significantly regulated proteins upon activation with ionomycin***

The activation leads to a regulation of most biological processes in particular signalling cascades, especially small GTPase mediated signal transduction. As expected, in conjunction with activation hemostasis, vesicle-mediated transport and cytoskeleton organization are heavily regulated as well. To fulfil the role of functional platelets, systems like adhesion and blood coagulation, were found to be regulated at the 15 min mark (seen in Table 3B).

| Table 3: Biological processes in which platelet proteins are mainly involved. | | |
|---|---|---|
| **A. Main biological processes realized by platelet proteins** | **Number of Proteins** | **Benjamini Score** |
| establishment of protein localization | 292 | 1,50E-57 |
| protein transport | 289 | 4,80E-57 |
| protein localization | 309 | 1,40E-52 |
| vesicle-mediated transport | 232 | 2,30E-50 |
| intracellular transport | 244 | 1,00E-45 |
| Golgi vesicle transport | 80 | 7,50E-31 |
| membrane organization | 144 | 1,20E-26 |
| intracellular protein transport | 140 | 2,10E-25 |
| cellular protein localization | 146 | 6,20E-24 |
| cellular macromolecule localization | 146 | 1,30E-23 |
| generation of precursor metabolites and energy | 117 | 6,00E-21 |
| negative regulation of protein metabolic process | 81 | 1,60E-18 |
| negative regulation of cellular protein metabolic process | 78 | 8,40E-18 |
| small GTPase mediated signal transduction | 109 | 9,30E-18 |
| actin filament-based process | 93 | 1,80E-17 |
| actin cytoskeleton organization | 88 | 9,20E-17 |
| proteasomal protein catabolic process | 54 | 1,40E-16 |
| proteasomal ubiquitin-dependent protein catabolic process | 54 | 1,40E-16 |
| hemostasis | 55 | 5,70E-16 |
| negative regulation of protein modification process | 58 | 8,10E-16 |
| positive regulation of ubiquitin-protein ligase activity | 42 | 3,70E-15 |
| **B. Main biological processes regulated after 15 min of activation with ionomycin** | **Number of Proteins** | **Benjamini Score** |
| small GTPase mediated signal transduction | 28 | 1,5E-13 |
| intracellular signaling cascade | 48 | 1,9E-10 |
| hemostasis | 16 | 9,2E-10 |
| regulation of body fluid levels | 17 | 2,9E-9 |
| coagulation | 15 | 3,7E-9 |
| blood coagulation | 15 | 3,7E-9 |
| vesicle-mediated transport | 28 | 1,5E-7 |
| wound healing | 17 | 1,7E-7 |
| response to wounding | 24 | 1,0E-5 |
| cell adhesion | 27 | 2,5E-5 |
| biological adhesion | 27 | 2,4E-5 |
| protein transport | 28 | 3,0E-5 |
| establishment of protein localization | 28 | 3,3E-5 |
| actin cytoskeleton organization | 15 | 4,7E-5 |
| actin filament-based process | 15 | 9,4E-5 |
| protein localization | 29 | 1,2E-4 |
| cell activation | 16 | 1,3E-4 |
| platelet activation | 7 | 1,4E-4 |

*Table 3:* ***The main biological processes in conjunction to expressed platelet proteins.***
*The determined biological processes, according to GO terms, in which platelet proteins are mainly involved at rest (A) and after 15 min of regulation (B). The number of proteins determined for each indicated biological function is indicated with the corresponding Benjamini score.*

For Table 4, this work will focus rather on the general conduct of the proteins in relation to activation, than on the proteins themselves, as the work of researching and grouping was done by other members of the group of Professor Christopher Gerner.

By comparing the protein profiles generated from lysate and secretome samples of untreated and treated platelets, a group of proteins emerges which is present inside the resting platelets and is released upon activation (red group). Several important platelet proteins and factors were released after the 15 min mark, as well as several growth factors.

Marked in green are proteins which are found downregulated inside the platelets as well as in the secretome. Should they not to be found at all in the secretome they are shown in black. These proteins seem to be under specific degradation processes, consequent to the rise of several proteases and upregulation of proteasomal proteolytic processes.

All these quite substantial changes reflect that the control samples were at a completely different status as the activated samples, supporting the claim of a successful NRP.

See next page:

*Table 4:* ***Differences in abundance of proteins in the analysed samples***
*Differences of LFQ values (logarithmic scale base two) determined in platelet lysates and secretomes of activated (15min and 3h of activation) versus resting platelets (con), with corresponding p-values (logarithmic scale base 10), are listed.*

| Acc.Nr. | Gene name | Protein name | lysate Δ_15min vs con | lysate p-value 15min vs con | lysate Δ_3h vs con | lysate p-value 3h vs con | secretome Δ_15min vs con | secretome p-value 15min vs con | secretome Δ_3h vs con | secretome p-value 3h vs con |
|---|---|---|---|---|---|---|---|---|---|---|
| P50995 | ANXA11 | Annexin A11 | -0.94 | 2.38E-08 | -1.05 | 2.38E-09 | 5.65 | 7.80E-10 | 5.35 | 2.66E-09 |
| P20073 | ANXA7 | Annexin A7 | -0.75 | 6.58E-10 | -0.67 | 4.75E-06 | 5.89 | 6.13E-10 | 5.62 | 8.53E-10 |
| Q15762 | CD226 | CD226 antigen | -1.23 | 1.61E-05 | -1.75 | 8.41E-04 | 2.88 | 5.71E-04 | 3.31 | 1.53E-04 |
| Q5SQ64 | LY6G6F | Lymphocyte antigen 6 complex locus protein G6f | -0.07 | 4.72E-01 | -0.79 | 5.96E-04 | 3.36 | 5.38E-08 | 4.51 | 7.99E-10 |
| Q9UIB8 | CD84 | SLAM family member 5 | -0.45 | 3.40E-03 | -1.33 | 2.07E-09 | 2.74 | 6.15E-10 | 3.37 | 1.83E-10 |
| P01133 | EGF | Pro-epidermal growth factor | -2.66 | 8.14E-09 | -2.59 | 1.84E-07 | 4.02 | 1.70E-05 | 3.51 | 4.07E-05 |
| O15117 | FYB | FYN-binding protein | -1.28 | 5.13E-09 | -1.93 | 1.02E-07 | 2.57 | 7.28E-04 | 2.86 | 2.42E-04 |
| P07359 | GP1BA | Platelet glycoprotein Ib alpha chain | -0.06 | 2.32E-01 | -0.85 | 3.36E-11 | 1.34 | 5.52E-08 | 2.04 | 2.86E-11 |
| P40197 | GP5 | Platelet glycoprotein V | -1.03 | 4.46E-12 | -1.08 | 2.65E-08 | 1.38 | 1.89E-06 | 1.21 | 5.67E-07 |
| Q9HCN6 | GP6 | Platelet glycoprotein VI | -2.79 | 6.95E-07 | -2.58 | 5.50E-09 | 3.45 | 1.42E-08 | 3.30 | 4.42E-09 |
| P02776 | PF4 | Platelet factor 4; CXCL4 | -1.81 | 2.75E-07 | -2.34 | 1.35E-06 | 0.79 | 1.86E-03 | 0.60 | 2.30E-02 |
| P10720 | PF4V1 | Platelet factor 4 variant | -2.71 | 3.61E-05 | -2.25 | 8.51E-04 | 4.79 | 1.24E-04 | 3.90 | 1.45E-03 |
| O95721 | SNAP29 | Synaptosomal-associated protein 29 | -6.10 | 1.60E-11 | -5.85 | 2.99E-10 | -1.81 | 3.58E-03 | -1.22 | 1.32E-02 |
| P37802 | TAGLN2 | Transgelin-2 | 0.00 | 9.78E-01 | -0.64 | 1.91E-07 | 0.76 | 1.40E-06 | 1.67 | 4.07E-11 |
| Q86YW5 | TREML1 | Trem-like transcript 1 protein | -1.20 | 5.95E-13 | -2.17 | 7.77E-15 | 1.90 | 2.75E-09 | 1.85 | 1.06E-09 |
| Q9Y6W5 | WASF2 | Wiskott-Aldrich syndrome protein family member 2 | -1.75 | 3.78E-06 | -1.57 | 6.41E-07 | 3.24 | 8.84E-06 | 3.22 | 8.72E-06 |
| Q14247 | CTTN | Src substrate cortactin | -9.44 | 1.96E-10 | -11.28 | 1.79E-14 | -3.24 | 3.51E-10 | -2.82 | 1.37E-07 |
| Q14847 | LASP1 | LIM and SH3 domain protein 1 | -4.66 | 9.03E-13 | -5.74 | 1.29E-09 | -1.27 | 1.75E-06 | -1.16 | 9.18E-05 |
| O00151 | PDLIM1 | PDZ and LIM domain protein 1 | -4.15 | 1.84E-16 | -5.19 | 1.12E-14 | -1.39 | 3.03E-07 | -0.99 | 1.51E-05 |
| Q9H4X1 | RGCC | Regulator of cell cycle RGCC | -5.48 | 1.18E-10 | -5.41 | 3.89E-10 | -1.64 | 1.62E-03 | -2.46 | 4.58E-06 |
| O00161 | SNAP23 | Synaptosomal-associated protein 23 | -3.05 | 6.20E-10 | -3.10 | 1.30E-10 | -1.92 | 2.53E-07 | -2.12 | 4.86E-07 |
| P37840 | SNCA | Alpha-synuclein | -3.13 | 4.95E-16 | -4.21 | 1.29E-10 | -3.73 | 2.65E-04 | -2.53 | 3.43E-06 |
| Q15942 | ZYX | Zyxin | -1.67 | 1.09E-07 | -1.82 | 1.36E-08 | -2.27 | 1.83E-08 | -2.14 | 5.06E-08 |
| Q08495 | DMTN | Dematin | -3.56 | 5.50E-16 | -3.85 | 1.79E-16 | - | - | - | - |
| Q99704 | DOK1 | Docking protein 1 | -5.43 | 5.00E-11 | -5.47 | 4.55E-10 | - | - | - | - |
| P50402 | EMD | Emerin | -8.24 | 1.87E-11 | -7.91 | 2.41E-11 | - | - | - | - |
| P27987 | ITPKB | Inositol-trisphosphate 3-kinase B | -7.98 | 1.62E-06 | -5.53 | 1.48E-03 | - | - | - | - |
| Q9UHB6 | LIMA1 | LIM domain and actin-binding protein 1 | -5.38 | 5.22E-08 | -5.59 | 3.30E-08 | - | - | - | - |
| Q7Z434 | MAVS | Mitochondrial antiviral-signaling protein | -5.75 | 6.70E-09 | -5.96 | 2.92E-09 | - | - | - | - |
| Q9P270 | SLAIN2 | SLAIN motif-containing protein 2 | -6.12 | 1.24E-14 | -5.57 | 5.45E-10 | - | - | - | - |
| P53814 | SMTN | Smoothelin | -6.17 | 5.30E-11 | -6.74 | 1.63E-13 | - | - | - | - |
| Q15836 | VAMP3 | Vesicle-associated membrane protein 3 | -5.58 | 2.03E-09 | -5.94 | 4.57E-08 | - | - | - | - |
| Q9BV40 | VAMP8 | Vesicle-associated membrane protein 8 | -4.94 | 6.07E-09 | -5.02 | 2.90E-09 | - | - | - | - |
| Q9UEU0 | VTI1B | Vesicle transport through interaction with t-SNAREs homolog 1B | -4.88 | 5.42E-09 | -3.98 | 1.34E-08 | - | - | - | - |
| O15231 | ZNF185 | Zinc finger protein 185 | -7.43 | 3.61E-10 | -7.60 | 4.49E-12 | - | - | - | - |
| P09525 | ANXA4 | Annexin A4 | -0.27 | 7.20E-03 | -0.22 | 8.92E-02 | 4.41 | 7.70E-07 | 4.17 | 6.99E-07 |
| P16671 | CD36 | Platelet glycoprotein 4 | 0.00 | 9.56E-01 | 0.02 | 8.39E-01 | 2.20 | 1.61E-07 | 2.17 | 2.89E-07 |
| Q9Y624 | F11R | Junctional adhesion molecule A | 0.35 | 2.59E-04 | 0.59 | 3.45E-06 | 3.56 | 1.53E-03 | 3.81 | 8.87E-04 |
| O95866 | G6B | Protein G6b | -0.41 | 1.76E-02 | -0.54 | 1.48E-04 | 2.38 | 2.10E-12 | 2.39 | 8.22E-12 |
| P19086 | GANZ | Guanine nucleotide-binding protein G(z) subunit alpha | 0.03 | 8.29E-01 | 0.50 | 7.71E-03 | 3.90 | 1.69E-05 | 3.56 | 4.09E-05 |
| P13224 | GP1BB | Platelet glycoprotein Ib beta chain | 0.16 | 5.47E-02 | 0.13 | 2.57E-01 | 1.47 | 1.45E-07 | 1.42 | 8.73E-07 |
| P14770 | GP9 | Platelet glycoprotein IX | 0.21 | 1.41E-02 | 0.16 | 6.41E-02 | 1.99 | 2.08E-07 | 1.90 | 3.44E-08 |
| P07948 | LYN | Tyrosine-protein kinase Lyn | 0.30 | 7.53E-04 | 0.22 | 2.60E-03 | 3.61 | 5.98E-05 | 3.86 | 3.66E-05 |
| Q13201 | MMRN1 | Multimerin-1 | -0.15 | 2.42E-02 | -0.18 | 2.52E-02 | 1.22 | 5.25E-12 | 0.94 | 1.64E-11 |
| Q5VY43 | PEAR1 | Platelet endothelial aggregation receptor 1 | -0.04 | 8.32E-01 | -0.04 | 7.06E-01 | 2.55 | 4.14E-04 | 2.31 | 9.53E-04 |
| P16284 | PECAM1 | Platelet endothelial cell adhesion molecule | 0.19 | 1.69E-02 | 0.27 | 1.54E-03 | 1.45 | 1.44E-06 | 1.39 | 1.68E-06 |
| Q15404 | RSU1 | Ras suppressor protein 1 | 0.24 | 7.13E-02 | 0.43 | 1.65E-02 | 0.80 | 5.48E-07 | 1.14 | 4.51E-12 |
| O75208 | COQ9 | Ubiquinone biosynthesis protein COQ9, mitochondrial | 1.92 | 1.00E-04 | 1.99 | 7.99E-05 | - | - | - | - |
| Q15125 | EBP | 3-beta-hydroxysteroid-Delta(8),Delta(7)-isomerase | 0.65 | 3.41E-01 | 1.91 | 3.00E-03 | - | - | - | - |
| Q8N766 | EMC1 | ER membrane protein complex subunit 1 | 1.73 | 4.88E-05 | 1.58 | 1.25E-03 | - | - | - | - |
| Q9H9P8 | L2HGDH | L-2-hydroxyglutarate dehydrogenase, mitochondrial | 0.95 | 3.20E-02 | 2.01 | 1.05E-04 | - | - | - | - |
| Q9BZF1 | OSBPL8 | Oxysterol-binding protein-related protein | 2.53 | 1.61E-07 | 2.54 | 1.19E-07 | - | - | - | - |
| P08559 | PDHA1 | Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial | 1.11 | 2.78E-03 | 1.46 | 3.23E-04 | - | - | - | - |
| Q9NSY2 | STARD5 | StAR-related lipid transfer protein 5 | 3.66 | 3.35E-05 | 1.99 | 8.89E-05 | - | - | - | - |
| P02786 | TFRC | Transferrin receptor protein 1 | 1.74 | 1.71E-04 | 1.20 | 5.67E-03 | - | - | - | - |
| Q9H1E5 | TMX4 | Thioredoxin-related transmembrane protein 4 | 0.37 | 5.26E-01 | 2.17 | 1.05E-04 | - | - | - | - |
| P84085 | ARF5 | ADP-ribosylation factor 5 | - | - | - | - | 1.11 | 8.83E-03 | 2.02 | 1.51E-04 |
| O95819 | MAP4K4 | Mitogen-activated protein kinase kinase kinase kinase 4 | - | - | - | - | 2.11 | 1.39E-09 | 2.24 | 6.79E-10 |
| P04085 | PDGFA | Platelet-derived growth factor subunit A | - | - | - | - | 1.34 | 5.80E-02 | 1.19 | 2.18E-02 |
| P01127 | PDGFB | Platelet-derived growth factor subunit B | - | - | - | - | 1.70 | 1.95E-04 | 0.84 | 2.11E-02 |
| P15153 | RAC2 | Ras-related C3 botulinum toxin substrate 2 | - | - | - | - | 3.93 | 1.35E-05 | 4.14 | 3.15E-06 |
| Q71RC9 | SMIM5 | Small integral membrane protein 5 | - | - | - | - | 2.19 | 4.93E-06 | 1.65 | 6.10E-05 |
| P49767 | VEGFC | Vascular endothelial growth factor C | - | - | - | - | 2.15 | 1.31E-03 | 0.84 | 2.70E-01 |

### 6.2.2. Indications of protein synthesis in platelets

Proteins in the colour blue in Table 4 show upregulation in the secretome upon activation, even though without significant decrease in the levels of the lysate. A possible explanation would be a considerable surplus concentration inside the cells.
Should the levels be roughly in the same range, a release would have been easily noticeable. Newly induced synthesis of proteins in platelets could be a reason of holding the concentration constant.
Another indication would be the proteins in violet, as the proteins were only found in the secretome of activated platelets, but neither in the lysates nor the secretomes of the control samples.

On the flipside several proteins were also exclusively detected in the lysates of activated platelets, highlighted in orange.

To round out this argument 80 proteins, involved in translation processes were detected in the platelets. Forty of these proteins were found significantly upregulated in the lysates of activated platelets.

## 6.3. Lipids

### 6.3.1. Identification of the regulated eicosanoids per shotgun analysis

As this series of experiments was started, it was not clear if eicosanoids could be analysed as a lot of challenges had to be overcome. The low concentrations, complex matrices, short active lifetimes, instability and other hurdles must be taken to refine the analytical processes. A major defiance is the chromatographic separation of isobaric molecules and ambiguous fragments and patterns in the MS/MS spectra, preventing a definite identification. Although through sometime painstaking comparison and precise comparison this work shows an orbitrap-based data-dependent shotgun eicosadomics method.
As this method was applied to the samples to determine excretion of eicosanoids, 26 were identified in the secretome (Table 5). One eicosanoid similar to Hepoxilin B3 was found. It

shows very alike fragmentation patterns with the same precursor mass, but the RT differs. Since there is no accurate match evident, an allocation was not feasible.

| Synonym | m/z | Systematic Name |
|---|---|---|
| 12S-HHTrE | 279.1966 | 12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid |
| 13S-HODE | 295.2279 | 13S-hydroxy-9Z,11E-octadecadienoic acid |
| EPA | 301.2173 | 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid |
| 11-HEPE | 317.2122 | 11-hydroxy-5Z,8Z,12E,14Z,17Z-eicosapentaenoic acid |
| 12-HEPE | 317.2122 | (+/-)-12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid |
| 12-oxo-ETE | 317.2122 | 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid |
| 15S-HETE | 319.2217 | 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid |
| 12R-HETE | 319.2217 | 12R-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid |
| 11S-HETE | 319.2217 | 11S-hydroxy-5Z,8Z,11E,14Z-eicosatetraenoic acid |
| 12S-HETE | 319.2217 | 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid |
| 9S-HETE | 319.2217 | 9S-hydroxy-5Z,7E,11Z,14Z-eicosatetraenoic acid |
| 11R-HEDE | 323.2592 | 11R-hydroxy-12E,14Z-eicosadienoic acid |
| HepoxilinB3 | 335.2228 | 10-hydroxy-11S,12S-epoxy-5Z,8Z,14Z-eicosatrienoic acid |

| | | |
|---|---|---|
| **HepoxilinB3-like** | **335.2228** | --- |
| **14-HDoHE** | **343.2279** | (+/-)-14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoicacid |
| **11ß-PGE2** | **351.2177** | 9-oxo-11S,15S-dihydroxy-5Z,13E-prostadienoic acid |
| **PGE2/8-iso-PGE2** | **351.2177** | 9-oxo-11R,15S-dihydroxy-5Z,13E-prostadienoic acid |
| **PGD2** | **351.2177** | 9S,15S-dihydroxy-11-oxo-5Z,13E-prostadienoic acid |
| **20-Hydroxy-LTB4** | **351.2177** | 5S,12R,20-trihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid |
| **13,14-dihydro-15-keto-PGD2** | **351.2177** | 11,15-dioxo-9S-hydroxy-5Z-prostenoic acid |
| **11β-PGF2α** | **353.2335** | 9S,11S,15S-trihydroxy-5Z,13E-prostadienoic acid |
| **8-iso-PGF2α** | **353.2335** | 9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid-cyclo[8S,12R] |
| **TXB2** | **369.2270** | 9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid |
| **Hepoxilin A3** | **335.2228** | 8-hydroxy-11S,12S-epoxy-5Z,14Z,9E-eicosatrienoic acid |
| **8-iso-15-keto-PGE2** | **349.202** | 9,15-dioxo-11R-hydroxy-5Z,13E-prostadienoic acid-cyclo[8S,12R] |
| **TXB1** | **371.2439** | 9S,11,15S-trihydroxy-thrombox-13E-enoic acid |

*Table 5:* ***Identified eicosanoids.***

Some of the lipids were already known for their involvement with platelet like hydroxyeicosatetraenoic acid (12S-HETE) and thromboxane B2 (TXB2). Others were not yet linked to platelets such as 9-HETE and 11-HETE.

To investigate the change in eicosanoid expression the relative levels were determined in the control samples as well as the activated ones, shown in Figure 9 and Table 6.

Most eicosanoids were strongly expressed already after the shorter amount of time; other like eicosapentaenoic acid (EPA) and 11-HEPE were newly induced upon platelet activation.

Some of the hydroxyeicosatetraenoic acids, like 12-HETE and 15-HETE seem to be pro-survival mediators, and are therefore associated with tumour progression.[50]

Another big information yield is the similarity in the patterns of the 4 different donors, apparent in the small error bars in Figure 9, as this information also shows that no platelets were more or less activated, showing the effectiveness of the established method of the NRP.

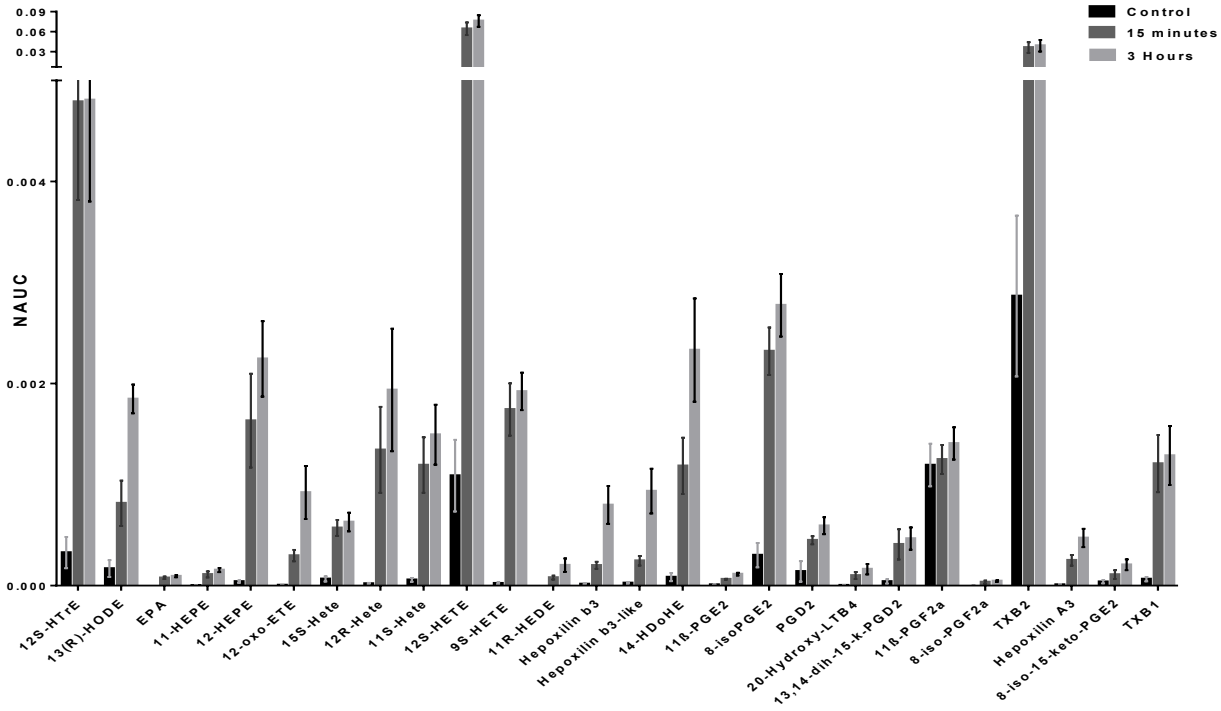


*Figure 9:* ***Regulation of eicosanoids in platelets upon activation with ionomycin for 15min and 3h.*** *Several eicosanoids were found massively upregulated in platelets upon activation with ionomycin. Proving their functional responsiveness and confirming therefore the functionality of the NRP. The data was normalized with the IS. The variation is over the different measurements, including biological (4 donors at 3 time points) as well as technical (three technical replicates).*

| Lipid | m/z | RT | Folds 15 min/ Control | P-value | Folds 3 h/ Control | P-value | Folds 3 h/15 min | P-value |
|---|---|---|---|---|---|---|---|---|
| 12S-HHTrE | 279.1966 | 15.87 | 14.64 | 1.76E-08 | 14.69 | 1.48E-08 | 1.00 | 9.25E-01 |
| 13S-HODE | 295.2279 | 19.36 | 4.82 | 2.52E-06 | 10.91 | 3.42E-11 | 2.26 | 1.88E-08 |
| EPA | 301.2173 | 20.89 | 4396.03 | 3.04E-10 | 5327.10 | 1.35E-11 | 1.21 | 3.46E-03 |
| 11-HEPE | 317.2122 | 18.22 | 47.79 | 8.99E-08 | 66.22 | 1.81E-11 | 1.39 | 1.62E-04 |
| 12-HEPE | 317.2122 | 18.71 | 40.59 | 1.27E-07 | 55.78 | 3.40E-10 | 1.37 | 7.66E-05 |
| 12-oxo-ETE | 317.2122 | 21.56 | 50.63 | 1.48E-09 | 157.01 | 1.06E-07 | 3.10 | 1.54E-05 |
| 15S-HETE | 319.2217 | 20.02 | 8.58 | 1.82E-09 | 9.47 | 1.02E-10 | 1.10 | 2.52E-01 |
| 12R-HETE | 319.2217 | 20.41 | 65.72 | 3.70E-07 | 94.73 | 2.79E-07 | 1.44 | 1.07E-03 |
| 11S-HETE | 319.2217 | 20.53 | 20.88 | 3.38E-08 | 26.15 | 4.18E-09 | 1.25 | 1.07E-03 |
| 12S-HETE | 319.2217 | 20.9 | 58.92 | 1.10E-10 | 69.70 | 5.24E-12 | 1.18 | 2.25E-03 |
| 9S-HETE | 319.2217 | 21.28 | 73.79 | 1.06E-10 | 81.34 | 7.89E-13 | 1.10 | 9.80E-02 |
| 11R-HEDE | 323.2592 | 23.15 | 112.03 | 1.93E-08 | 285.06 | 4.12E-07 | 2.54 | 1.19E-05 |
| Hepoxilin b3 | 335.2228 | 17.68 | 12.65 | 4.71E-10 | 50.22 | 1.43E-08 | 3.97 | 4.50E-07 |
| Hepoxilin b3-like | 335.2228 | 17.91 | 9.49 | 1.34E-09 | 36.10 | 1.77E-08 | 3.80 | 4.32E-07 |
| 14-HDoHE | 343.2279 | 20.78 | 14.07 | 2.51E-08 | 27.68 | 5.67E-09 | 1.97 | 3.27E-07 |
| 11ß-PGE2 | 351.2177 | 7.06 | 6.75 | 2.66E-12 | 12.11 | 5.35E-11 | 1.79 | 8.72E-09 |
| PGE2/8-iso-PGE2 | 351.2177 | 7.41 | 7.69 | 3.57E-12 | 9.20 | 2.74E-12 | 1.20 | 1.87E-05 |
| PGD2 | 351.2177 | 8.04 | 3.19 | 1.84E-08 | 4.21 | 3.09E-10 | 1.32 | 4.03E-07 |
| 20-Hydroxy-LTB4 | 351.2177 | 8.65 | 23.83 | 1.51E-06 | 38.70 | 4.81E-07 | 1.62 | 1.01E-07 |
| 13,14-dih-15-k-PGD2 | 351.2177 | 9.34 | 9.86 | 2.93E-06 | 11.25 | 1.91E-08 | 1.14 | 1.86E-03 |
| 11β-PGF2α | 353.2335 | 6.87 | 1.05 | 7.99E-02 | 1.18 | 1.58E-06 | 1.13 | 1.12E-08 |
| 8-iso-PGF2a | 353.2335 | 7.14 | 41.20 | 5.92E-06 | 52.61 | 2.17E-10 | 1.28 | 9.68E-03 |
| TXB2 | 369.2270 | 6 | 12.59 | 5.28E-08 | 13.56 | 4.43E-08 | 1.08 | 5.92E-03 |
| Hepoxilin A3 | 335.2228 | 15.52 | 24.37 | 3.71E-09 | 45.91 | 1.67E-09 | 1.88 | 9.26E-09 |
| 8-iso-15-k-PGE2 | 349.202 | 8.25 | 2.83 | 1.21E-05 | 5.33 | 1.08E-08 | 1.88 | 2.15E-08 |
| TXB1 | 371.2439 | 6.01 | 18.70 | 4.90E-08 | 19.93 | 3.29E-08 | 1.07 | 1.87E-03 |

*Table 6:* ***The identified eicosanoids and their regulatory effect in the platelet secretome***
*26 eicosanoids were identified in platelet supernatants. This Table shows their masses (m/z values), retention times (RT) and changes in abundance between platelets activated for 15min or 3h and controls, including their corresponding p-values.*

### 6.3.2. Correlation of the eicosanoid and protein regulation

As the information of the proteomic and lipidomic experiments is combined it is possible to imagine pathways as platelets are activated with ionomycin (Figure 10).

The combination of information surrounding the interconnectivity of proteins and enzymes were also generated by members on the team of Professor Gerner and are stated here to complete and round out the argument.

It seems that calcium mobilization triggers two independent signals inside the platelets, which leads to the cleavage of arachidonic acid (AA), eicosapentaenoic acid (EPA) and linoleic acid (LA) from diacylglycerols. One by the activation of calcium release-activated calcium channel

protein 1 (ORAI1) and the other by targeting RAS guanyl-releasing protein 2 (RASGRP2) both leading to more calcium influx into the platelets.

The PUFAs are metabolized to other eicosanoids which are released into the extracellular matrix. Most of the eicosanoids stem from AA and are HETES and PGs, but also hepoxilins and thromboxanes were found.

Interestingly, eicosanoids found upregulated upon platelet activation in our experiments have not only pro-coagulation functionalities, but several of them have anti-coagulation effects, as indicated in Figure 10.

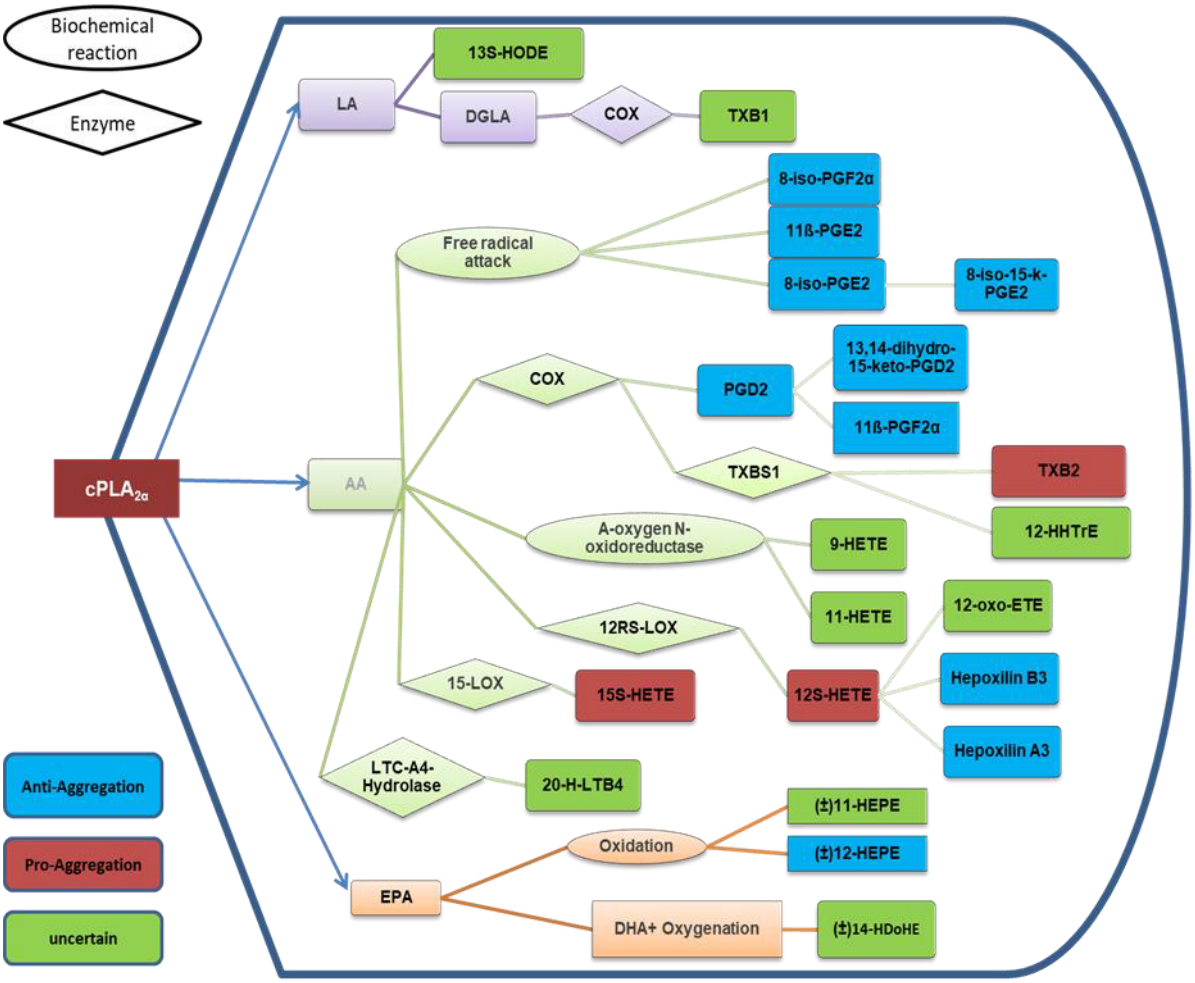


*Figure 10:* ***Eicosanoid production pathways in platelets upon activation with ionomycin.*** *Combination of the data of proteomics and lipidomics experiments. Indications of pro- or anti-coagulation characteristics were gathered per literature search. Identified molecules are shown in black; transparent are molecules which were not found, presumably as they are below the LOD.*

## 7. Discussion

In this work the proteome and eicosanoid alterations of platelets upon activation were examined.

For this endeavour it was necessary to develop a method to keep platelets in a resting state outside the body. A process to isolate the blood cells from taking whole blood to the measurement of the proteins and lipids was optimized.

The method did not use any platelet inhibitors such as prostaglandin E1. It gives the opportunity for comparison of idle and activated platelets, giving the differences from a reference point.

In examining platelet lysates as well as secretomes separately it is possible to see which molecules are present in the cells and those which are released by the cells.

Another point is using two different time points after activation, 15 min and 3 h respectively, giving an overview over the highly dynamic processes that platelets are involved in and are highly necessary for the survival of the body.

One of the hypotheses established in the introduction of this work was the observation that platelets are actively synthesizing proteins when getting activated by calcium mobilization. As platelets are anucleate, this seems counterintuitive at first. First claims where published in the 1960s by Warshaw et al.[51].Several papers where since written about different possible mechanisms, including using mRNA and ribosomes[52], [53], instilled as a legacy of megakaryocytes from which they are originally derived.[54]

This work also came to this conclusion as we see a strong induction of platelets protein synthesis upon activation. Comparison of the protein distribution and shift in lysates and secretomes makes the observation possible which proteins are designated to be secreted and as some include potential tumour promoters should be under close surveillance in the future.

Thanks to the setup of the experiment, it was possible to not only observe newly synthesized proteins, but also regulated ones. It showed that upregulated proteins at activation were categorized at pro-aggregation. This highlights one of the most important points of this work, the ability to keep platelets in an inactivated state to ensure a NRP. The NRP therefore allows the platelets to be fully responsive to activation.

An interesting part is the finding of proteins being subjected to degradation processes and an upregulation of proteases. This seems to be a necessary step for the pro-coagulation activities of platelets.[55] To counteract uncontrollable escalation of the aggregation and therefore thrombosis, degradation processes seem to be highly necessary. Subsequently, activation must be a highly regulated and fine-tuned process. Activation by any agonist must thus induce not only pro-coagulation but also anti-coagulation events to ensure a smooth regulation and coming back to rest.

Fortunately, the eicosanoids showed a similar pattern in reaction to activation like the proteins, reinforcing the legitimacy of the NRP. Several lipid levels were massively increased upon activation. It seems that even the mild activation of ionomycin leads to the expression or regulation of the known mediators of aggregation and that the expression of eicosanoids allows the platelets to fine tune coagulation.

The activation of the blood cells leads to a release of AA, EPA and LA, the starting materials of all the subsequent metabolites, from platelet membrane phospholipids through the activation of $cPLA_{2\alpha}$. As already mentioned at the beginning of this work AA can be metabolized in different pathways. Through the COX pathway, AA is metabolised by Platelet prostaglandin G/H synthase 1 (PTGS1 or COX1) via the instable prostaglandin H2 (PGH2), which is further metabolized to the stable form PGD2 which has anti-aggregation effects. PGD2 can itself undergo different metabolism pathways, either into the isoprostane 11β-PGF2α or into 13,14-dihydro-15-keto-PGD2. All of those eicosanoids can be classified as anti-aggregation and were all upregulated in the experiments. [56], [57], [58]

PGH2 can also be metabolized by thromboxane-A synthase (TBXAS1) into thromboxane A2 (TXA2), the major eicosanoid aggregation mediator produced by platelets, which is very unstable and was not found. It functions as a precursor for the more stable TXB2. [59]

TBXAS1 is also able to metabolize PGH2 into 12-hydroxy-heptadecatrienoic acid (12S-HHTrE). Apparently, there is an additional non-enzymatic way for 12S-HHTrEto be synthesized even in case of TBXAS1 inhibition, which is a survival mechanism of the platelets.[60] Both, TXB2 and 12S-HHTrEwere found massively upregulated upon treatment of platelets with ionomycin.

Another eicosanoid class the HETEs are also synthesized from AA. Arachidonate 12-lipoxygenase, 12S-type (ALOX12) metabolizes AA into 12-HETE (S and R forms) which, depending on the context, model and stimuli, can have pro or anti-aggregation effects.[61] As

our model involves calcium mobilization as an aggregation stimulus, it is highly probable that in our experiments 12-HETE has a pro-aggregation effect as it is described by Nyby et al..[62]

12S-HETE may also promote tumour cell growth and metastasis[40], underlining the same tumour-promoting capabilities of activated platelets as described above in case of secreted proteins, like growth factors. Interestingly, 12-HETE is metabolized into hepoxilins which exert an anti-aggregation effect.[63]

The other found HETEs, 15S-HETE seem to have pro-aggregation effects[64], while 11-HETE and 9-HETE are not yet known for their role in platelets, although the later seem to be linked to pro-inflammation and obesity.[65]

Free radical-catalyzed peroxidation of AA led to the formation of isoprostanes (8-iso-PGE2, 8-iso-15k-PGE2, 11b-PGE2 and 8-iso-PGF2α) in activated platelets. Those are reported to have an anti-aggregation effect[66].It needs to be addressed that 8-iso-PGE2 and its metabolites can also play a pro-inflammatory role depending on the nature of the platelet activating stimuli[67].

Finally, we also observed upregulation of 11-HEPE, 12-HEPE and 13-hydroxy-octadecadienoic acid (13-HODE) in activated platelets. These metabolites result from cleavage of EPA or LA by $cPLA_{2\alpha}$. EPA is metabolized via oxidation into HEPE products; 12-HEPE is reported to have anti-aggregation effects[68].

LA, on the other hand, is metabolized by a lipoxygenase into 13-HODE, which may have pro- or anti-aggregation effects, depending on the concentration.[69]

Important to note is that all eicosanoids were induced in platelets already after 15 minutes of activation, and that those levels were maintained until the second measurement after 3 hours of activation (Figure 9).

This leads to the conclusion that there seems to be a strong and sudden start of eicosanoid synthesis in platelets upon stimulation, whereas after this first step, no further regulation seems to occur.

## 8. Conclusion and Outlook

This work tried to answer three main questions and successfully did so.

Is it possible to establish a method to obtain platelets in a mostly inactive state, the NRP, and can it be supported by the data generated by proteomics and lipidomics?
Is the concept of newly induced synthesis of proteins as suggested by the former SD-PAGE experiments supported by the more complex proteome profiling data?
Is it possible to establish methods to extract, concentrate and analyse eicosanoids on a qualitative and quantitative level?

All three questions can now be answered positively, however as often this work can only be seen as a stepping stone to build understanding and indicate directions for further studies. New information lead to new questions, in this case it would be beneficial to investigate the PTMs of the proteins and examine quantitative proteomics and lipidomics.
Further research into eicosanoids and other lipids is necessary as it is known, that they change their roles in relation to others, be it each other, proteins, lipids and other metabolites. Another aspect that seems to be highly influential is the localisation of the expressed biomolecules as well.
Continued study of the issue would be of high interest as the activated platelets may serve as tumour promoting entities releasing growth factors as well as eicosanoids with pro-survival functions. It is also of note that platelets seem to play a role in a high quantity of diseases, some with urgent and high impact to society.

Something that this work illustrates quite well is the immense velocity in which analytical advancements take place. As ideas stemming from one PAGE-experiment now lead to robust data of thousands upon thousands of proteins, show what was inconceivable years ago. These new methods highlight how every tiny step is important and needs attention. It becomes clearer that bioinformatics continues to be essential as it is not possible to master the number of generated data manually any more. On the flipside, especially young disciplines like metabolomics are still malleable and harbour nearly unfathomable possibilities.

This work shows therefore one of the old principles; all good ideas get their time, a few just a bit later.

# 9. Lists of Figures and Tables

## List of Figures



## List of Tables

## 10. References


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