Systems Biology based studies on anti-inflammatory compounds

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Concluding remarks and future perspectives

The studies performed in this thesis show that holistic analytical strategies, based on transcriptomics, proteomics and metabolomics in combination with multivariate data analysis tools are very powerful in finding specific markers for cellular processes and effects of biologically active compounds. This approach was used to categorize anti-inflammatory drugs based on their mRNA, protein and/or lipid expression patterns. The outcome of the categorization depends on the availability and choice of compounds that can be used as reference compounds in the experiment. The approach that was developed enabled us to classify zilpaterol as a β_2-adrenergic receptor agonist based on a transcriptomic and metabolomic analysis. In addition, although we could not fully elucidate the mechanism of the anti-inflammatory effect of *Cannabis sativa* extracts, we were able to show that this mechanism does not overlap with that of several known anti-inflammatory compounds, including corticosteroids, MAP kinase inhibitor, β_2-adrenergic receptor agonist, and proteasome inhibitor. Moreover, our results suggest that unheated and heated Cannabis extracts use a different mechanism to exert their anti-inflammatory action.

The transcriptomics data showed to be the most suitable dataset to categorize anti-inflammatory drugs, using the U937 cell line. The categorization was less successful when the proteomics data of the applied 2-D gel technology was used. Microarray technology is a straight-forward method. In one experiment ten thousands of genes (from approx. 30.000) can be analysed simultaneously. The possibility to measure almost all genes at once makes transcriptomics a favourable method for holistic approaches at this moment.
Proteomics methods are less suitable for holistic approaches because in a proteomics experiment only a fraction of the total number of proteins in a biological sample (approximately 1000 out of estimated 50.000-500.000 proteins) with more or less the same physiological characteristics (e.g. concentration range, hydrophobicity,) can be observed simultaneously. These proteins are often high abundant proteins, while low abundant proteins that are important in many metabolic pathways, e.g. those involved in the inflammatory response, are not readily seen. Hence the chance of missing crucial regulatory proteins, e.g. those involved in the action of anti-inflammatory drugs, is present. A more in-depth investigation of the proteome is therefore necessary. Pre-fractionation of the sample and enrichment strategies (e.g. immunoaffinity chromatography, subcellular fractionation, and sequential extraction) will improve the chance to find interesting proteins other than the

common ‘housekeeping’ proteins, as was demonstrated by analysing the secreted protein fraction in Chapter 4. Applying more than one proteomics technique (e.g. 2-D gel electrophoresis, LC-MS, protein arrays) within a proteomics study will improve the recovery of a wide range of diverse proteins. None of the above-mentioned proteomics techniques is preferable over the other, instead these techniques are complementary. The analysis of protein samples separated into many different fractions and the application of different analytical techniques makes the analysis of only one sample a time-consuming task. Nevertheless, the protein level remains one of the most interesting levels to investigate biological functions and disease states. In practice it is therefore advisable to focus solely on a specific part of the proteome that is closely related to the biological question, instead of analysing the whole proteome. For example, when there is an interest in G-protein coupled receptors, the membrane fraction has to be investigated rather than the whole cell lysate. For this reason, in future research it is important to focus on pre-fractionation techniques that are reproducible and yield high protein content.

Another important issue in proteomics is protein identification. Insufficient amounts of peptides being generated after in-gel digestion and their low signal intensity makes it difficult to identify proteins with high confidence. These drawbacks have to be improved especially when low abundant or proteins with low molecular masses have to be identified. With LC-MS methods this problem is less pronounced but in some methods the proteins have to be identified using a minimal number of peptides, especially in cases where peptides have been tagged and subsequently pre-fractionated (e.g. cysteins) for quantification purposes. Moreover, information on post-translational modifications, such as phosphorylation, are easily missed. Furthermore, mass accuracy is an important issue in protein identification. The more accurate the peptide mass, the larger the chance that a protein or peptide will be identified with high confidence. The introduction of the Fourier transform mass spectrometer (FT-MS), a high resolution mass spectrometer, in the proteomics platform will significantly improve protein identification.

The analysis of the metabolome is hampered by similar problems as encountered during proteome analysis. The diverse properties and vast concentration ranges of molecules forces the investigator to use many different methods to analyse, if possible at all, the whole metabolome. Using only the lipid data we were able to categorize the anti-inflammatory drugs based on their mechanisms of action. The separation by PC-DA could not be assigned to one

or more specific biomarkers. This means that the combined regulation of several lipids is responsible for the differences found in the lipid expression patterns by PC-DA. This underlines the power of multivariate data analysis tools to find hidden correlations and trends in large datasets.

Multivariate data analysis tools proved to be powerful in classifying samples according to their differentiation state (Chapter 2), anti-inflammatory effect (Chapter 3) and disease state (inflammation, inhibition of inflammation) in Chapter 4. Moreover, the use of multivariate data analysis tools enabled us to find specific biomarkers for differentiation (Chapter 2), anti-inflammatory effects of different anti-inflammatory drugs (Chapter 3) and biomarkers that were linked to anti-inflammation and the β_2-adrenergic receptor (Chapter 4). The datasets obtained by proteomics, transcriptomics and metabolomics was in most cases based on a limited number of samples, compared to the number of variables. Multivariate data analysis tools are mathematical tools, and therefore, finding false positive results can not be excluded, especially when the number of data points is at the lower limit. It is therefore important to regard the statistical tools employed as explorative tools and all findings have to be biologically validated, for example by using traditional biochemical methods like real time PCR, Western blot, or immunoassays. When the observed differences can not be validated univariatly, other more comprehensive methods have to be used (e.g. a comparison of the transcriptome with the proteome of the same sample). In any case, the modern technologies used in transcriptomics, proteomics and metabolomics research yield vast amounts of data, and the statistical tools currently available are not optimally designed to meet this challenge. Therefore, extensive validation of any result found by statistical analysis is essential. Hopefully, in the future statisticians and biologists can find more suitable methods to cope with these large datasets. Statisticians have to develop methods that can cope with a smaller number of samples and biologists have to find a way to reduce the number of variables by filtering the datasets to remove the 'noise'. Unfortunately, it is not possible to filter the datasets by using univariate data analysis tools, because these tools do not take into account the possible correlations between two or more variables. Although the use of smaller datasets or microarrays which contain less genes could be an alternative to overcome these issues, but this may possibly narrow down the research to already well known pathways. This contradicts with the idea of the holistic approach of systems biology, which aims to investigate all cellular pathways, and to identify the web of interactions between these pathways.

In this thesis we implemented parts of the systems biology circle (Fig 1) in our investigations and demonstrated the benefits of this approach. For example, by using holistic methods we were able to categorize anti-inflammatory drugs and discovered biomarkers for β_2-adrenergic receptor agonists which may be involved in some of the adverse affects of these compounds in the treatment of asthma. In the future, systems biology will become more and more important in the discovery of drugs, and investigation of diseases.

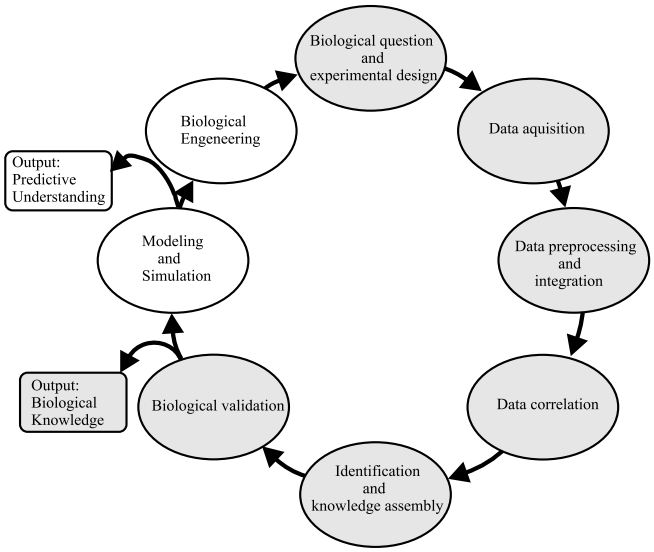

Figure 1 Systems Biology cycle with the different interchangeable modules. The modules used in this thesis are highlighted (grey).

It is becoming increasingly apparent that a single biomarker cannot provide sufficient information and specificity, and that more complex biomarker profiles are often necessary to elucidate the action of drugs or to characterize a disease. But there are still numerous challenges to deal with in systems biology. Complete mapping of all possible pathways and networks of one particular system to build a computer-run mathematical model will be an enormous task. Therefore, close collaboration of scientific experts in many different disciplines (e.g. transcriptomics, proteomics, metabolomics, biochemistry, biostatistics, bioinformatics, molecular biology, computer modelling) are required for the successful implementation of systems biology in drug discovery and disease studies. Until now, many of the results obtained with systems biology approaches are actually hypotheses, which have to be validated biologically by traditional genetic or cell-biological methods. Another issue is the accessibility of databases. At this moment, most labs involved in systems biology have their own databases, and publicly available systems biology data is limited. It is very important that a willingness to share this knowledge will develop. Different databases can be integrated in a meaningful way. The need for standardized annotations and lab procedures are

of great importance. Minor variations in procedures, reagents, or environment may already have a great impact on the biological system under investigation.

Systems biology is a powerful tool but the success of this technology can be greatly enhanced by an efficient cooperation of many scientists of different disciplines, as well as of the universities and companies involved in systems biology.

Summary

The introduction of the 'omics' techniques (transcriptomics, proteomics, and metabolomics) and systems biology, has caused fundamental changes in the drug discovery process and many other fields in the life science area. In this thesis we explored the possibilities to apply these holistic technologies to investigate the effects of known and potential anti-inflammatory compounds on macrophages. For this purpose we made use of a monocyte-like human histocytic lymphoma cell line U937. U937 cells can be induced by phorbol 12-myristate 13-acetate (PMA) to undergo differentiation into a macrophage-like phenotype. The two differentiation stages, monocyte and macrophage, were compared by using oligonucleotide microarrays and 2-D gel electrophoresis in combination with principal component analysis (PCA). This differentiation study is described in Chapter 2. The differential expression of three protein biomarkers, gamma interferon inducible lysosomal thiol reductase (GILT), cathepsin D and adipocyte-fatty acid binding protein (A-FABP) were biologically validated by Western blot and real time polymerase chain reaction (real time PCR). GILT and A-FABP were also found to be differentially expressed at the mRNA level as indicated by the results of the microarray experiment. Moreover, the transcriptomics data revealed a large number of additional putative differentiation markers in U937 macrophages, many of which are known to be expressed in peripheral blood-derived macrophages. From the results presented in Chapter 2 can be concluded that the U937 cell line is an excellent model system for the blood-derived macrophage and that microarrays and 2-D gel electrophoresis are suitable methods to identify biomarkers for differentiation.

Chapter 3 describes the use of a systems biology approach to categorize anti-inflammatory drugs based on their mRNA, protein and lipid expression pattern, as determined by oligonucleotide microarrays, 2-D gel electrophoresis and a LC-MS method for lipids, in combination with principal component discriminant analysis (PC-DA). The results described in this chapter demonstrate that different classes of anti-inflammatory compounds show distinct and characteristic mRNA, protein, and lipid expression patterns, which can be used to categorize known anti-inflammatory drugs, as well as to discover and classify new leads. The latter was exemplified by the categorization of zilpaterol, a poorly characterized β_2-agonist. Exposure to zilpaterol gives rise to an almost identical expression pattern as that observed after exposure to the well-characterized β_2-agonists clenbuterol and salbutamol, suggesting that zilpaterol is indeed a β_2-agonist. In addition, this study revealed potential biomarkers for

the different anti-inflammatory drugs under investigation. The categorization of the anti-inflammatory drugs on the basis of proteomics data alone was not successful. The most likely explanation for this is that by the analysis of whole cell lysates, only highly abundant proteins can be visualized, while the low abundant proteins, which are often involved in important metabolic pathways, are not. Therefore, a more focused approach was used to investigate the mechanism of action of zilpaterol, which is described in Chapter 4.

In Chapter 4, U937 macrophages were stimulated with LPS to induce an inflammatory response. This response was inhibited by the addition of zilpaterol (LZ) and this inhibition was antagonized by the β_2-adrenergic receptor antagonist propranolol (LZP). Two-dimensional difference gel electrophoresis (DIGE) in combination with Student's *t*-test and two multivariate data analysis tools (PCA and partial least squares discriminant analysis PLS-DA) were used to examine the secreted proteome induced by the three treatments. This revealed 8 potential protein biomarkers. The protein spots were identified using nano LC-MS-MS. Only two of the identified proteins, namely macrophage inflammatory protein-1β (MIP-1β) and macrophage inflammatory protein-1α (MIP-1α) are known to be secreted proteins. The inhibition of MIP-1β by zilpaterol and the involvement of the β_2-AR and cyclic adenosine-3',5'-cyclic monophosphate (cAMP) were confirmed using a specific immuno-assay. The experiments described in this chapter demonstrate the importance of pre-fractionation of complex protein samples before performing proteomics studies.

The categorization of zilpaterol in Chapter 3 as a β_2-adrenegic receptor agonist was further explored in Chapter 5. In this chapter we investigated the binding affinity of zilpaterol to the β_1- and β_2 receptor by using a receptor binding assay. Furthermore, we examined the role of the β_1- and β_2 adrenoceptor in the inhibition of the LPS induced tumor necrosis factor-alpha (TNF-α) production and the induction of cAMP by U937 macrophages. For this purpose we made use of a selective β_1-receptor antagonist (atenolol), a selective β_2-antagonist (ICI 118551) and a non-selective β-antagonist (propranolol). Finally, the inhibitory effect of zilpaterol on the TNF-α production was investigated in LPS-treated male Wistar rats. The results obtained in this way clearly show that zilpaterol is a β_2-adrenergic agonist and a inhibitor of the LPS-induced TNF-α production by macrophages both *in vivo* and *in vitro*.

The three β_2-agonists specific biomarkers, Granulocyte Chemotactic Protein-2 (GCP-2/CXCL6), Oncostatin M (OSM), and Vascular Endothelial Growth Factor (VEGF) that were

identified in Chapter 3, were further examined in Chapter 6. The three markers were significantly up-regulated both in U937 macrophages and in blood-derived macrophages exposed to a β_2-agonist (clenbuterol and zilpaterol) in the absence or presence of LPS, as determined by a specific enzyme-linked immunosorbent assays (ELISA). Moreover, this up-regulation was also accomplished by other cyclic AMP elevating agents (forskolin, prostaglandins E_2, and dibutyryl cAMP), suggesting a role of cAMP in the up-regulation of GCP-2/CXCL6, VEGF and OSM. We hypothesize that these proteins may be involved in some of the adverse effects in the treatment of asthma with β_2-adrenergic receptor agonists.

In the second part of this thesis we focussed on a multi-component drug, namely *Cannabis sativa*. In Chapter 7, the immuno-modulating effects of unheated and heated Cannabis extracts were investigated. This study revealed that unheated Cannabis extracts and its major non-psychoactive compound Δ^9-tetrahydrocannabinolic acid (THCa) were able to inhibit the LPS induced TNF-α production both in U937 macrophages and in blood-derived macrophages. The inhibitory effect on TNF-α was not mediated by the cannabinoid receptors CB_1 and CB_2. Furthermore, this study showed that unheated Cannabis extracts and THCa exert their inhibitory effect on the TNF-α production via a mechanism that is different from that of heated Cannabis extract and its main constituent the psychoactive compound Δ^9-tetrahydrocannabinol (THC). The inhibition of TNF-α release by unheated Cannabis extract and THCa was prolonged over a relatively long period of time. By contrast, although THC and heated extracts initially inhibit the release of TNF-α, after longer incubation times they seem to increase TNF-α production to levels that are even higher than in the absence of THC or Cannabis extract. This difference in response of the U937 macrophages to THC and THCa was also observed in an experiment in which we examined the effects on phosphatidylcholine specific phospholipase C (PC-PLC) activity. Unheated Cannabis extract and THCa inhibited the PC-PLC activity in a dose-dependent manner, while THC induced PC-PLC activity at high concentrations. Finally, we studied the effect of THCa and unheated Cannabis extract in a pilot study using an Experimental Autoimmune Encephalomyelitis (EAE) mouse model. Unheated Cannabis extract and THCa had a favourable effect on the clinical and histological signs of EAE. However, these results are preliminary and not clearly significant, therefore further investigation is necessary.

Chapter 8 describes the categorization of unheated and heated Cannabis extracts using the same model system as described in Chapter 3. The mRNA patterns obtained from U937

macrophages exposed to LPS in the absence or presence of different anti-inflammatory drugs and unheated and heated Cannabis extracts were analysed using PC-DA. The study revealed that heated and unheated Cannabis extracts give rise to different expression patterns, which is in agreement with the observations made in Chapter 7 that they exert their TNF-α inhibitory effect via different pathways. Moreover, their expression patterns did not overlap with that of other classes of anti-inflammatory compounds known to inhibit the TNF-α production. These results suggest that the Cannabis extracts can not be assigned to one of the above mentioned classes of inflammatory inhibitors. Further investigation is necessary to unravel the exact mechanism of action of unheated and heated Cannabis extracts.

In conclusion, the studies in this thesis show that the application of systems biology approaches are very useful in the categorization of anti-inflammatory compounds based on their mRNA and lipid expression patterns and to find specific biomarkers for these compounds. The categorization based on the protein expression pattern was less successful. This is most probably due to the fraction of proteins that was analysed on the gel. With proteomics techniques only a small fraction of proteins can be analysed simultaneously. Pre-fractionation, enrichment techniques and different analytical methods are therefore necessary to analyse a wide range of proteins with diverse physiological properties and dynamic range. The datasets obtained by transcriptomics, proteomics and metabolomics were analysed using statistical and pattern recognition tools. The datasets often contained a limited number of samples with respect to the large number of variables. It is therefore important to use these techniques as an explorative tool only and to validate the potential biomarkers found by additional individual measurements.
Taken together, the use of systems biology for the investigation of anti-inflammatory drugs yielded very promising results, even though only a small part of the systems biology circle (Fig. 1) was used.

Samenvatting

De ontwikkelingen op het gebied van genomics, systeembiologie, en de verschillende afzonderlijke 'omics'-disciplines (transcriptomics, proteomics en metabolomics) hebben ingrijpende veranderingen te weeg gebracht in het biomedisch onderzoek. Dit geldt zeker ook voor het onderzoek en de ontwikkeling van nieuwe geneesmiddelen.
Bij het onderzoek beschreven in dit proefschrift werden onder andere de mogelijkheden bestudeerd om deze 'holistische' technieken toe te passen bij studies naar de eigenschappen van bestaande en nieuwe ontstekingsremmende verbindingen. In de studies werd gebruik gemaakt van de U937 cellijn, welke is afgeleid van humane monocyten. U937 cellen kunnen na blootstelling aan phorbol 12-myristate 13-acetaat (PMA) differentiëren naar een cel met een macrofaag-achtig fenotype. De twee differentiatiestadia (monocyte en macrofaag) werden met elkaar vergeleken door middel van oligonucleotide microarrays en 2-dimensionale gelelectroforese in combinatie met principale component analyse (PCA). Deze differentiatiestudie wordt beschreven in hoofdstuk 2. De differentiële expressie van drie eiwit biomarkers, gamma interferon inducible lysosomal thiol reductase (GILT), cathepsin D en adipocyte-fatty acid binding protein (A-FABP) werden biologisch gevalideerd met behulp van Western blot en real time polymerase chain reaction (real time PCR). De inductie van GILT en A-FABP werd ook op mRNA niveau gevonden met behulp van microarrays. Naast deze markers leverde het microarray experiment nog meer mogelijke biomarkers op, waarvan er diversen al beschreven waren voor perifere bloedmacrofagen. De resultaten beschreven in hoofdstuk twee laten zien dat de U937 cellijn een geschikt modelsysteem is voor de uit bloed geïsoleerde macrofaag en dat microarrays en 2-D gelelectroforese geschikte technieken zijn om differentiatie biomarkers te identificeren.

Hoofdstuk 3 beschrijft een systeembiologische aanpak om ontstekingsremmende verbindingen te karakteriseren op basis van hun mRNA-, eiwit- en lipide- expressiepatroon. Deze expressiepatronen werden geanalyseerd met behulp van oligonucleotide microarrays, 2-D gelelectroforese en een LC-MS methode voor lipiden, in combinatie met principale component discriminant analyse (PC-DA). De resultaten gepresenteerd in dit hoofdstuk laten zien dat verschillende klassen van ontstekingsremmers verschillende en karakteristieke mRNA-, eiwit- en lipide- expressiepatronen induceren, die gebruikt kunnen worden om bekende en nieuwe ontstekingsremmers te classificeren. De methode werd getoetst met behulp van zilpaterol, een nog relatief weinig beschreven en gekarakteriseerde β_2-agonist. De

blootstelling van U937 macrofagen aan zilpaterol resulteerde in een bijna identiek expressiepatroon als wanneer de U937 macrofagen blootgesteld werden aan klassieke β_2-agonisten. Daarnaast leverde deze aanpak mogelijke biomarkers op, voor de verschillende klassen ontstekingsremmers gebruikt in het experiment. Het karakteriseren van ontstekingsremmers op basis van de proteomics resultaten alleen was minder succesvol. De meest waarschijnlijke verklaring hiervoor is dat wanneer er gekeken wordt naar extracten verkregen uit hele cellen, alleen de eiwitten die in sterke mate tot expressie komen zichtbaar zijn. De eiwitten die in lage concentratie aanwezig zijn en vooral betrokken zijn bij belangrijke metabole routes worden hierdoor overschaduwd. Daarom werd in hoofdstuk 4 een meer gefocuste methode toegepast om de werking van zilpaterol te bestuderen.

In hoofdstuk 4 werden U937 macrofagen blootgesteld aan LPS om een ontstekingsreactie te induceren. Deze ontsteking werd vervolgens geremd door de toevoeging van zilpaterol. De werking van zilpaterol werd weer opgeheven door de toevoeging van een β_2-antagonist, propranolol. De DIGE technologie (difference gel electrophoresis) in combinatie met de Student's *t*-test en twee multivariate data analyse methoden (PCA en partial least squares discriminant analysis (PLS-DA)) werden gebruikt om de uitgescheiden eiwitfractie van de hierboven beschreven behandelingen te bestuderen. Deze methode leverde 8 mogelijke biomarkers op. De eiwitten werden met behulp van nanoLC-MS geïdentificeerd. Slechts 2 van de 8 eiwitten werden geïdentificeerd als een uitgescheiden eiwit, namelijk macrophage inflammatory protein-1beta (MIP-1β) en macrophage inflammatory protein-1alpha (MIP-1α). De remming van MIP-1β door zilpaterol en de betrokkenheid van de β_2-receptor en cyclic adenosine-3',5'-cyclic monophosphate (cAMP) werd bevestigd met behulp van een immunoassay. De experimenten beschreven in dit hoofdstuk laten zien dat het voor proteomics studies van belang is om complexe eiwitmonsters vooraf te pre-fractioneren.

De karakterisering van zilpaterol als een β_2-agonist in hoofdstuk 3 werd verder onderzocht in hoofdstuk 5. In dit hoofdstuk werd eerst de bindingsaffiniteit van zilpaterol tot de β_1- en β_2-receptor bepaald met behulp van een receptor bindingstudie. Vervolgens werd de betrokkenheid van de β_1- en β_2-adrenoceptor bestudeerd bij de remming van de tumor necrosis factor alpha (TNF-α) productie door cellen blootgesteld aan LPS. Hiervoor werd gebruik gemaakt van een selectieve β_1-receptor antagonist (atenolol), een selectieve β_2-antagonist (ICI 188551) en een niet-selectieve β-antagonist (propranolol). Als laatste werd het remmende effect van zilpaterol op de TNF-α productie onderzocht in mannelijke Wistar ratten

behandeld met LPS. De verkregen resultaten laten zien dat zilpaterol een β_2-agonist is en een potente remmer van de LPS-geïnduceerde TNF-α productie door macrofagen *in vivo* en *in vitro*.

Drie β_2-agonisten biomarkers geïdentificeerd in hoofdstuk 3, Granulocyte Chemotactic Protein-2 (GCP-2/CXCL6), Oncostatin M (OSM) en Vascular Endothelial Growth Factor (VEGF) werden verder onderzocht in hoofdstuk 6 met behulp van specifieke immunoassays. De drie markers werden significant geïnduceerd in U937 macrofagen en in bloedmacrofagen door β_2-agonisten (clenbuterol en zilpaterol) in de aan- en afwezigheid van LPS. De inductie van deze markers werd ook bewerkstelligd door andere cAMP verhogende verbindingen (forskolin, prostaglandine E2 en dibutyryl cAMP). Deze resultaten suggereren een mogelijke rol van cAMP in de inductie van GCP-2/CXCL6, OSM en VEGF. Ook wordt een mogelijke rol van deze eiwitten bediscussieerd bij enkele nadelige bijeffecten die kunnen optreden tijdens de behandeling van astma met β_2-agonisten.

In het tweede deel van dit proefschrift werd een geneesmiddel bestudeerd dat uit meerder bio-actieve componenten bestaat, namelijk *Cannabis Sativa*. In hoofdstuk 7 werd het immuun-modulerende effect van onverhit en verhit Cannabis extract onderzocht. Deze studie laat zien dat zowel onverhitte Cannabis extracten als de voornaamste component die in de plant aanwezig is, Δ^9 – tetrahydrocannabinolic acid (THC-zuur) de productie van LPS geïnduceerde TNF-α productie kan remmen in U937 macrofagen en in bloedmacrofagen. THC-zuur is zelf niet psychoactief en de cannabinoid receptors CB1 en CB2 bleken niet betrokken te zijn bij het TNF-α remmende effect. Verder kwam uit deze studie naar voren dat onverhitte Cannabis extracten en THC-zuur hun TNF-α remmende effect via een ander mechanisme bewerkstelligen dan verhitte Cannabis extracten. Dit gold ook voor het hoofdbestanddeel van verhit Cannabis, de psychoactieve component Δ^9-tetrahydrocannabinol (THC). Onverhit Cannabis extract en THC-zuur remde de TNF-α productie gedurende een langere tijd, terwijl verhit Cannabis extract en THC de TNF-α productie na langere tijd juist stimuleerden tot een niveau dat hoger was dan verkregen na blootstelling aan LPS alleen. In de studie werd ook gekeken naar het effect van Cannabis op de phosphatidylcholine specifieke phospholipase C (PC-PLC) activiteit in U937 macrofagen. Hierbij werd een belangrijk verschil gevonden tussen THC en THC-zuur. Onverhit Cannabis extract en THC-zuur remde de PC-PLC activiteit op een concentratie afhankelijk manier, terwijl THC bij hogere concentraties juist de PC-PLC activiteit stimuleerde. Uiteindelijk werd het effect van onverhit Cannabis extract en

THC-zuur bestudeerd in een EAE (Experimental Autoimmune Encephalomyelitis) muis model. Onverhit Cannabis extract en THC-zuur vertoonden een gunstig effect op de klinische en histologische verschijnselen van EAE. De *in vivo* waargenomen effecten waren echter niet eenduidig en verder onderzoek is hier noodzakelijk.

Hoofdstuk 8 beschrijft de karakterisering van onverhitte en verhitte Cannabis extracten met behulp van het modelsysteem dat beschreven staat in hoofdstuk 3. De mRNA expressiepatronen van U937 macrofagen blootgesteld aan LPS in de aan- en afwezigheid van verschillende ontstekingsremmende verbindingen en verhitte en onverhitte Cannabis extracten werden geanalyseerd met behulp van PC-DA. De studie laat zien dat verhit en onverhit Cannabis extract een verschillend mRNA expressiepatroon induceert. Dit is in overeenstemming met de resultaten van hoofdstuk 7, waar werd gevonden dat onverhitte en verhitte Cannabis extracten op verschillende manieren de TNF-α productie remmen. Bovendien vertoonden de mRNA expressiepatronen geen overlap met de expressiepatronen van bekende klassen van ontstekingsremmers, waarvan bekend is dat zij de TNF- α productie remmen. Dit zou kunnen betekenen dat de Cannabis extracten niet ingedeeld zouden kunnen worden bij de in dit onderzoek gebruikte klassen van ontstekingsremmers. Meer onderzoek is nodig om de precieze werkingsmechanismen van onverhitte en verhitte Cannabis extracten te ontrafelen.

Uit de studies beschreven in dit proefschrift kunnen we concluderen dat toepassing van systeembiologie een belangrijke bijdrage leveren aan het ontdekken en karakteriseren van ontstekingsremmende verbindingen, wanneer de mRNA- en lipide- expressiepatronen en het vinden van specifieke biomarkers worden geïntegreerd. De resultaten verkregen uit de proteomics studie waren minder goed bruikbaar voor het classificeren van ontstekingsremmers, waarbij gebruik werd gemaakt van het U937 modelsysteem. Een mogelijke verklaring hiervoor zou kunnen zijn dat er slechts een beperkte eiwitfractie op de 2-D gel geanalyseerd kon worden. Prefractionering, eiwit-verrijkingstrategieën en de toepassing van verschillende analytische methoden tegelijkertijd zijn daarom nodig om een grotere verscheidenheid aan eiwitten, met verschillende fysiologische eigenschappen en dynamisch bereik te kunnen analyseren. De transcriptomics, proteomics en metabolomics datasets werden geanalyseerd met behulp van patroonherkenningsmethoden. De datasets bevatten vaak een gelimiteerd aantal monsters ten opzichte van het aantal variabelen. Het is daarom

belangrijk om deze methoden slechts verkennend te gebruiken. Daarnaast moeten de gevonden biomarkers biologisch gevalideerd worden door middel van additionele metingen.

Ten slotte dient te worden opgemerkt dat bij het hier beschreven onderzoek nog maar een deel van de methoden en technieken uit de systeembiologie cirkel is toegepast (figuur 1, hoofdstuk 9). Desondanks vormen de resultaten van het onderzoek een onderbouwing voor de stelling dat de principes en de aanpak van de systeembiologie het onderzoek en de ontwikkeling van nieuwe geneesmiddelen blijvend zullen beïnvloeden.