Lipid droplets as dynamic organelles of fat deposition and release: Translational research towards human disease

Rendicontazione

Informazioni relative al progetto

LIPIDOMICNET

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Final Report Summary - LIPIDOMICNET (Lipid droplets as dynamic organelles of fat deposition and release: translational research towards human disease)

Executive summary:

The consortium invested major efforts to develop new high-throughput (HT) and high content mass spectrometry (MS) technologies for the analysis of all aspects of lipid metabolism, from lipid classes to individual lipid species. Lipidomic methods were extended to the simultaneous analysis of mono / polyglycerophospholipids, cholesterol precursors, oxysterols, phytosterols and glycosphingolipids. These new methods in conjunction with newly developed bioinformatics tools will have impact beyond the project to stimulate discoveries in preclinical and clinical science and lipid related disorders. The use of mouse models provided mechanistic insight into the aberrant regulation of lipid storage and release dynamics in diabesity. A tissue and nucleic acid bank was generated using different animal models to delineate the pathways that are conserved between humans and mice to better understand diabesity and steatotic liver disease. Several pathways were newly identified playing an important role in both liver and adipocyte lipid processing. The identified genes, transcripts, proteins and lipids may contribute to altered lipid droplet (LD) biogenesis in human diseases. Combining transcriptomics and lipidomics with newly developed bioinformatic analysis tools led to the identification of functional keynodes in gene regulatory networks that contribute to altered LD biogenesis. Collection and analysis of human patient material, especially liver, adipose tissue and macrophages focused on disorders of endolysosomal (phospholipidosis) and LD (foam cells) storage and release. About 80 LD genes were identified in adipocytic cell lines in a genome-wide gene suppression approach combined with fluorescent lipid storage analysis. Many proteins resembled LD apolipoproteins, lipid hydrolases and transferases, and proteins involved in lipid transfer, ER-stress processes and autophagy. Analysis of patient cohorts identified and characterised key genes such as ATP10D involved in diabesity. Study of HCV-infected liver cells, adipocyte differentiation and the cross talk between visceral and subcutaneous adipose tissue identified novel genes associated with diabesity.

The lipid stress response of macrophages, granulocytes and cardiomyocytes was investigated at the molecular level to understand endolysosomal LD storage and / or processing. Transcriptomic, proteomic and lipidomic profiles were studied in normal and mutant cells with monogenetic alterations of lipid traffic.

In addition, to better understand lipid stress-signalling in atherosclerotic lesions, the impact of lipids on monocyte / macrophage differentiation was studied. Novel methods for isolation and multi-omic characterisation of cells, extracellular vesicles (EVs) and LD were developed.

New technologies were established and existing technologies improved for the analysis of lipid entry into cells. Lipid probes modified by stable isotopes, fluorescence, or chemical reporters were prepared and applied to characterise lipid entry pathways and the impact of different proteins (e.g. ORP family). Human diseases associated with endolysosomal lipid storage (phospholipidosis) induced by modified lipoproteins, drugs, and genetic defects were further characterised. The LIPIDOMICNET wiki (see http://www.LIPIDOMICNET.org online) was made public and all standard processing procedures (SPPs) are available online. New lipid-related pathways have been made available at resources such as Reactome, EndoNet and the Proteome database. A framework for the storage of MS-based lipidomics data has been developed, containing a standard nomenclature for lipid species coming from MS, a database of theoretically existing lipid species and software for correlation of lipidomic, transcriptomic, micro ribonucleic acid (miRNA) and proteomic data.

Project context and objectives:

The LIPIDOMICNET project aimed to exploit the recent developments in lipidomics to establish HT methods and define druggable targets and novel biomarkers related to LD composition. The consortium studied lipid-protein interactions and investigated the dynamics of fat deposition and release in relevant cells, genetic mouse models and materials from human diabesity cohorts as hallmark of energy overload diseases with major health care impact in Europe. The results have been integrated across the different discovery platforms to enhance and annotate the pathway knowledge as found in the literature.

As defined in the work programme, the following major scientific and technological objectives of this highly focused science and technology (S&T) project were set:

- develop HT tools allowing the discrimination of LD heterogeneity as related to lipid and protein composition, interaction, and function, to validate the known constituents and identify new components;
- expand the current structural and dynamic knowledge of LD assembly and disassembly and to identify novel lipidomic and proteomic constituents and interactions thereof;
- to dissect non-clathrin and clathrin mediated lipid-influx pathways and to characterise their abnormalities towards phospholipidosis development in relevant lipid storing cells and tissues;
- to validate the known LD-efflux pathways and identify novel proteomic and lipidomic targets, related to druggability and biomarker development;
- to extend the knowledge of cellular lipid- and protein kinases and phosphatases, regulating influx, efflux and storage in endylysosomes (phospholipidoses) and LD, and to identify novel extracellular signals that affect lipid storage and release;
- to identify and validate transcriptional networks regulating influx/efflux of lipids and dynamics of LD assembly and disassembly;
- to perform translational research from mouse to man applied to the LD theme in liver cells, adipose tissue, macrophages and granulocytes during metabolic overload dependent transdifferentiation, and to determine the relationship between LD and hepatitis C virus (HCV) infection;

- to develop LIPIDOMICNET as a Wiki format public database that provides the connectivity algorithms to synergise knowledge and data analysis generated by lipidomic, proteomic, genomic, and transcriptomic HT and high-content (HC) detection;
- to establish a world-leading European consortium on the basis of genuine and equal partnership between leading academic groups, analytical chemists, cell biologists, clinical scientists, bioinformatics, computer scientists, and biostatisticians to provide a resource for further knowledge in lipidomics for the benefit of the European Community and for the improvement of health care.

WP1 - Management

WP1 aimed to achieve successful management of the consortium activities. Management work package (WP) members supported the beneficiaries through all reporting periods mediating optimal communication between European Commission (EC) and the consortium. Several main objectives such as ensuring of efficient communication within and beyond the consortium at different levels served this main goal. For this purpose, the advanced tools such as LIPIDOMICNET Wiki homepage, audio-video conferences (Skype, Webex, Oovoo, etc), phone- and Skype-consultations, sharing and cloud services were regularly employed.

WP2 - Enabling technologies

WP2 bundled technology development as an important part of the project to enable progress in all project areas. A number of enabling technologies were developed, reaching from single molecule tracing and high resolution imaging over mass spectrometric lipid analysis with high sample turnover, to automated chemometric and biometric analysis and integration of data. Strategically, there were some tasks that needed a dedicated project section whereas others appeared to be better integrated into the context of data production. Accordingly, the advanced imaging technologies were financially supported directly within WP2, whereas lipid MS, proteomics, transcriptomics and others were embedded into various subgroups. As a horizontal communication structure, task forces were established that organised the contact between groups working on related problems. All technologies, both newly developed and improved or standardised, were carefully evaluated and then disseminated within the consortium, but also to the interested public, by placement on the technology section of the LIPIDOMICNET wiki page and allowing external scientists to participate in our workshops and meetings.

WP3 - Mouse models of liver and adipose LD processing

WP3 aimed to establish mouse models to assess nutritionally and genetically controlled settings for regulators of formation, metabolism and degradation of LD in vivo and in cell culture models. The overall context of the LIPIDOMICNET project aimed at development and exploitation of lipidomics technology for identification of druggable targets and biomarkers related to LD metabolism and composition. Within this frame of objectives, translational research from mouse to human was carried out in the course of the project. Priority was given to cells from adipose and liver tissues under physiologic and pathological situations. The demand for integration within LIPIDOMICNET partners afforded close cooperation with WP2 under the aspect of development of HT MS technology for lipidomic analysis and, by the same token, with WP7 for data integration and deposition, as well as their interpretation by bioinformatic approaches.

All this cooperation was mandatory for gaining insights into biochemical mechanisms leading to aberrant LD morphology and overall metabolism in mouse and human. Consequently, close cooperation with WP4 was instrumental on arriving at models best suited for understanding the human pathophysiological situation in Diabesity and steatotic liver disease.

WP4 - Dysregulation of liver and adipose LD processing in human diseases

WP4 was directed towards determining pathways regulating LD formation and turnover and how these respond to different physiological and pathophysiological states. To this end WP4 established, integrated and collected patient cohort material for in vitro testing. The data generated in WP4 were compared and contrasted with the data generated in WP3 so that the experimental benefits of mouse to man studies were maximised. By adopting a small interfering RNA (siRNA) screening approach using model cell lines and lipidomic, proteomic and transcriptomic analysis of the patient cohort material WP4 has identified target genes and pathways which will have the potential for druggability and use for biomarkers. The analysis of LD-regulating signalling pathways aimed to extend our knowledge of cellular lipid and protein kinases and phosphatases regulating influx, efflux and storage in LDs and to identify the extracellular signals governing this regulation. Building upon this work WP4 adopted a translational research approach from mouse to man in liver cells, adipose tissue and in collaboration with WP5 in other cell types such as macrophages. These studies focused on metabolic overload-dependent transdifferentiation and on the relationship between LD formation and HCV infection.

WP5 - LDs and lipid bodies in human macrophages and leukocytes

WP5 investigated the dynamics and role of lipids in macrophage and granulocyte biology at the molecular and functional level and looked into the monocyte-macrophage differentiation process. The requirements of lipids for monocyte-macrophage differentiation were investigated and the underlying transcriptional mechanisms uncovered. Lipidomic changes in the plasmalogen and sphingolipid composition of the differentiating cells were correlated with changes in their gene expression pattern. The identified patterns were analysed for their functional properties and evaluated for their use as clinical biomarkers. Using bioinformatics correlation analysis, the underlying regulatory networks were analysed. Furthermore the molecular mechanisms of endolysosomal lipid storage (phospholipidoses) and lipid-droplet formation in macrophages were studied. For this purpose monocyte-derived macrophages were challenged with the modified lipoproteins enzymatically degraded LDL (Eldl) and oxidised LDL (oxLDL) and the cellular lipid storage pattern visualised by fluorescence microscopy as well as performing lipidomic and transcriptomic analysis. Cells from Niemann-Pick type C (NPC) disease patients were used to further characterise endolysomal cholesterol export, phospholipidosis and intracellular lipid trafficking. Special emphasis was put on BMP and cardiolipin species as endolysosomal and mitochondrial stress reporter molecules. The effects of lipid unloading using HDL and apoAI were studied in vitro in monocyte-derived-macrophages. The oxLDL induced LD response was also investigated under normoxic and hypoxic conditions and the role of PAT family proteins in macrophages during development of insulin resistance, in atherosclerosis and during chronic myocardial ischemia was studied. Chronic and acute exposure to modified lipoproteins also influences leukocyte (PMN) lipid homeostasis and induces paracrine cell signalling. To address this subject, novel methods such as nanoparticle tracking analysis (NTA) were established and evaluated. Here direct effects of lipoproteins on granulocytes (PMN) were compared with effects on monocyte-

derived-macrophages. EVs from granulocytes were charactersed in their lipidomic, miRNA and proteomic properties and functional aspects in atherosclerotic plaque maintenance studied. EVs were also isolated from platelet preparations compared to the lipid and protein compositions of PMN-EV and evaluated as surrogate markers for platelet function.

WP6 - Cellular lipid entry pathways into LDs and lamellar bodies

WP6 was related to cellular lipid entry pathways into endolysosomal lamellar bodies and LDs. The major tasks were to establish and to apply methods for analysing lipid entry into different types of cells. A major focus was on the role of lipid binding proteins in this process; especially the role of different ORPs (oxysterol binding protein-related proteins), NPC proteins, and the function of saposins has been clarified with the aid of cells deficient in relevant proteins, the production of recombinant proteins, and the development of new tools for their analysis. The characterisation of lipotoxic drugs and their role in LD formation has been addressed with the aid of lipid probes that have been modified by fluorescence, isotopes, and other structural modifications. MS has been applied for the characterisation of lipid uptake pathways, and its application to lipidomics has been extended.

WP7 - Bioinformatics

WP7 aimed to maximise the value of the data generated throughout the project, and also at providing new software tools for the processing, analysis and storage in the specific case of lipidomics data. Task forces consisting of domain experts and bioinformaticians defined SPPs and defined the corresponding standard operating procedures (SOPs) for the different data types used in the project.

The task forces developed a new nomenclature system and a data format standard to allow correct reporting and storage of MS (MS)-based lipidomics data. As a key point, the results coming from different platforms were integrated into the LIPIDOMICNET wiki and other publicly available databases / resources. The wiki concept was employed to allow and encourage annotations and comments from the community at large. These were all essential objectives to have the basic building blocks to provide a basic bioinformatics infrastructure for MS-based lipidomics data. In addition, a lipid identifier reference system was also created and is available via a new resource called 'LipidHome'. Furthermore, the curation of lipid-related pathways was one of the main objectives of this WP. This information has been incorporated into resources such as Reactome, EndoNet or the ProteomeTM databases. An innovative tool for biochemical pathway tracking of non-labelled and stable isotope labelled lipid species (analysed by MS) was developed, including features such as the visualisation of the direction and significance of concentration changes in lipid species. The work performed in Regensburg was further integrated into the BioUML. The BioUML platform was used to build processing pipelines taking advantage of the information available in a number of public databases / repositories, including the ones enriched in the context of LIPIDOMICNET.

WP8 - Dissemination and training

WP8 enhanced communication tools being continuously optimised in parallel to newest developments was planned to be used in the project. In close cooperation with WP1, WP8 members together with the project manager and integrated investigators disseminated project related information to consortium members

and (when was applicable) to general public, organising meetings, conferences, workshops and exchange visits.

Efforts have been made to prepare and publish special issues on focused themes of the project. For example, devoted to central themes of LIPIDOMICNET, a special issue of Chemistry and Physics of Lipids entitled 'Analysis and function of oxysterols and other regulatory and lipotoxic molecular lipid species' was published in 2011 and is available from ScienceDirect web-page: http://www.sciencedirect.com/science/journal/00093084/164/6 Reports on dissemination strategies from all partners have been collected in cooperation with the management team and WP leaders. WP8 assisted the involved partners in promotion, dissemination and public release of the achievements of the consortium. WP8 ensured extensive dissemination activities taking place within the network of involved institutions as well as beyond. Amongst others, integration of partners, active involvement and training of young scientists and collection of publications were also objectives and subsequently, achievements of WP8.

Overall progress within LIPIDOMICNET is twofold:
i) generation of novel data which informs about the related issues of human health; and are
ii) available as a platform for wider understanding of the role of lipid storage and release in health and diseases.

Project results:

Lipids are central to the regulation and control of cellular processes by acting as basic building units for biomembranes, the platforms for the vast majority of cellular functions. The recent developments in lipid MS have set the scene for a completely new way to understand the composition of membranes, cells and tissues in space and time by allowing the precise identification and quantification of alterations of the total lipid profile after specific perturbations.

LIPIDOMICNET addressed LDs as dynamic organelles with regard to composition, metabolism and regulation. Lipid storage and release in multiple cells and tissues leads to transdifferentiation of multiple organs creating, fatty liver, obesity, white muscle and macrophage foam cells which are the hallmark of all energy overload diseases. The project exploited recent advances in lipidomics technology to establish HT methods to define drugable targets and novel biomarkers related to LD lipid and protein species, their interaction and regulation during assembly, disassembly, storage and release. The research groups of various backgrounds and interested have been united in six WPs (WPs 2 to 7) to study lipid protein interactions and investigate the dynamics of fat deposition and release in relevant cells as a hallmark of energy overload diseases with major health care impact in Europe.

Work progress and achievements during the period for WP2

Imaging technology: the focus in this project section was development of methods for LD imaging beyond the standard fluorescence imaging using commercially available dyes. A common problem of many LIPIDOMICNET groups, detection of LDs in living or fixed cells, was addressed with the development of LD540, a novel platalet LD dye with improved specificity and stability. LD540 is a lipophilic dye, based on

the Bodipy fluorophore, for microscopic imaging of LDs. In contrast to previous LD dyes, it can be resolved spectrally from both green and red fluorophores allowing multi-colour imaging in both fixed and living cells. Due to its slightly higher lipophilicity the dye can be used at about 10-fold lower concentrations than the popular Bodipy493, resulting in a significantly lower unspecific membrane background staining. Its improved specificity, brightness and photostability supports live cell imaging, which was used to demonstrate by two-colour imaging LD motility along microtubules. The dye is now in use by many LIPIDOMICNET groups and numerous other groups all over the world.

For biological systems that either do not fluoresce or cannot tolerate the toxicity associated with staining and the photo-bleaching of fluorophores, their intrinsic chemical properties can be used as contrast mechanisms through coherent anti-Stokes Raman scattering (CARS), To illustrate the method's potential the consortium gave a subcontract to Dr A. Volkmer at the University of Stuttgart to study the uptake mechanism of the two atherogenic model lipoproteins, E-LDL and Ox-LDL, into human macrophages. In summary, this study has demonstrated quantitative hyperspectral CARS imaging for the non-invasive and label-free study of Ox-LDL and E-LDL induced LDs in living cells. Without a priori knowledge, the full Raman spectrum is recorded for each image voxel with high spatial resolution (sub-femtoliter volume) and with fast acquisition times in the order of milliseconds per voxel. As an example, quantitative chemical structure analysis of intra-organelle lipids has been demonstrated by mapping their degree of acyl chain unsaturation.

Major efforts were devoted to advanced electron microscopy and have led to significant improvements in EM immunocytochemistry of lipid structures like lipoproteins and LDs, in EM-tomographic analysis of LDs and in EM lipid tracing using click chemistry. These methods are particularly useful to study the complex organisation and the rapid dynamics of hepatic lipid metabolism and have direct impact on the work in other WPs, in particular WP5 and WP6.

Using freeze fracture electron microscopy, possible pathways of LD growth were studied. Homotypic fusion of small LDs to create larger ones, is one proposed mechanism though the evidence for this process continues to be debated. Possible fusion has been suggested from live cell fluorescence imaging, but the low resolution and other technical factors, such as vertical movement of structures out of the viewing plane, have prevented definitive conclusions to be reached. While thin-section electron microcopy has adequate resolution, extraction of lipids together with the surface phospholipid monolayers of closely apposed droplets during processing creates false fusion-like images. Freeze-fracture electron microcopy offers some advantages over other approaches. Freeze-fracture typically reveals the LDs as closely associated but discrete spheroid or ovoid bodies with a characteristic layered structure. These features help define each droplet as an individual entity. Despite being so tightly pact that individual droplets appear to touch and even become flattened by mutual pressure at such contact regions, the boundaries of the individual droplets are normally quite distinct, suggesting a strong resistance to fusion. Only occasionally is a possible site of continuity between the contents of apposed droplets seen. Whether such sites represent droplets caught in the act of fusion or the fracture path has simply failed to be deviated sufficiently to show the boundaries clearly at these sites cannot be determined with certainty. It is nevertheless striking that, from the appearance observed in freeze-fracture, the larger LDs frequently appear to be aggregates of smaller droplets. The overwhelming impression created by these images is that numerous smaller LDs have amalgamated to create a larger one. One of the theoretical objections to fusion as a mechanism of

LD growth is that, owing to the reduction in overall surface area, an excess of surface monolayer would result that could not be accommodated via the mechanisms operating in membrane-bound vesicles.

MS

Large amounts of different methods for mass spectrometrical lipid analysis were either newly developed or were improved and standardised or automated high throughput analysis. For bile acid species quantification, a simple, sensitive liquid chromatography-tandem MS (LC-MS/MS) method for the analysis of bile acid profiles in human plasma/serum was developed. Validation was performed according to FDA guidelines and overall imprecision was below 11 % Coefficients of variation (CV) for all species. This method is currently under DAkkS accreditation. Also, a novel LC-MS / MS method for the rapid, simultaneous quantification of sphingolipid metabolites including sphingosine, sphinganine, phyto-sphingosine, di- and trimethyl-sphingosine, sphingosylphosphorylcholine, hexosylceramide, lactosylceramide, ceramide-1-phosphate and dihydroceramide-1-phosphate was developed. In contrast to most published methods based on reversed phase chromatography, hydrophilic interaction liquid chromatography (HILIC) achieved good peak shapes, a short analysis time of 4.5 min and most important co-elution of analytes and their respective internal standards. Also this method was validated according to FDA guidelines and showed excellent precision, accuracy, detection limits and robustness.

Furthermore, a reliable GC-MS/MS method for the simultaneous determination of cholesterol precursors, plant sterols, and oxysterols was developed. The main advantage of the method is the rapid (8 min) and simultaneous determination of a set of cholesterol precursors, plant sterols, oxysterols, and osyphytosterols with a short analysis time and excellent peak resolution. The hyphenation of GC and tandem MS has successfully enhanced the performance of approved GC methods, both in analytical sensitivity and in time savings. Because the main applications of sterol profiling in human plasma can be seen in neurodegenerative diseases, atherosclerosis, and cholesterol metabolism disorders, we excluded the implementation of cholesterol epoxides, and other autoxidative species. The method offers a valuable tool for direct therapeutic stratification by discriminating with a single analysis hyperabsorber from hypersynthesizer patients. Furthermore, a sterol profile identifies metabolic overload dependent uncoupling of cholesterol biosynthesis. The method contributes to the diagnosis of some rare genetic diseases like cerebrotendinous xanthomatosis, NPC disease or Smith-Lemli-Opitz-Syndrome in a single run. For High throughput lipidomic analysis, in a close collaboration between several partner groups, a novel quaternary pump HPLC chromatographic system was developed which allows the following classes of lipids to be separated prior to infusion into an MS: TAG, DAG, MAG, FFA, Ceramide, sphingosine, S1P, LPA, LPC, LPI, LPE, PC, PI, PE, PA, PG, BMP, Cardiolipin, sphingomyelin. Use of this has allowed us to analyse > 700 molecular species in a single run. The system has been charactersed and is now generating analytical data from adipose and liver tissue samples, as well as various cell lines.

This analysis system is complemented by an integrated platform for lipidomic analysis at the level of molecular species covering the workflow from sample preparation to data analysis. The analytical core is 2D HPLC coupled to ion cyclotron resonance MS. The platform enables analysis of a broad range of lipids within one chromatographic run combined with identification of minor compounds present in amounts as low as 0.01% of base peak. Furthermore it allows for fast profiling of lipid molecular species with regard to up and down regulation in search for lipid biomarkers. Flexibility is even further broadened with suitable

internal standards added to samples. In this case the platform affords quantitative determination of the lipidome.

A special highlight is the analysis of phosphoinositides by MS. There has been a long standing difficulty with determining the phosphoinositides using MS. There are a number of reasons for this: the phosphoinositides are signalling molecules, consequently there generation and removal is tightly regulated and the more highly phosphorylated forms particularly PtdIns3,4,5P3 are short lived entities; additionally the phosphate groups on the inositol ring are labile and lost or they migrate around the inositol ring during extraction; the phosphoinositides are strongly charged and thus bind to cellular proteins making extraction difficult; the highly charged lipids also bind to glass and steel making recoveries low. A number of methods have been reported, though none have been reproducible and easily adaptable. We have addressed the problem by adopting a derivatisation procedure which has the advantage that it stabilises the lipids and by shielding the charges reduces non-specific losses. Lipids are isolated from cell or tissue extracts and then the free phosphate groups are methylated using trimethylsilyldiazomethane. Following partial purification on a C4 reverse phase column, the diacyl groups of the lipids are identified following neutral loss of 598 amu. This allows the identification of distinct PtdIns3,4,5P3 species, a similar approach can identify PtdInsP2 though it is not possible at this stage to fully characterse PtdIns4,5P2 from PtdIns3,4P2 or PtdIns3,5P2. Inclusion of appropriate standards allows semi-quantification.

Raman and CARS microspectroscopies

The underlying hypothesis for this project has been the unique diagnostic capability of Raman and CARS microspectroscopies in offering non-invasive, label-free, and quantitative in-situ chemical analysis of lipid species with high spatial resolution (sub-femtolitre volume) and chemical structure information. The main project objectives consisted of providing access for the LIPIDOMICNET consortium to these highly experimental technologies and to the relevant know-how in both experiment and data analysis. The specific aim has been the demonstration of the full potential of these enabling technologies in lipidomic research.

The work was broken down into three collaborative sub-projects that reflect different research areas currently investigated by partners P01 and P10 of the LIPIDOMICNET consortium, which resulted in the following accomplishments:

The non-invasive identification of 7-Ketocholesterol as one of the lipid oxidation products in atherogenic oxidised lipoproteins (in collaboration with University Hospital Regensburg, G. Schmitz, P01):

A. The non-invasive chemical mapping of LD composition in living human macrophages (in collaboration with University Hospital Regensburg, G. Schmitz, P01).
B. The Raman-spectroscopic characterisation of polyene lipids as versatile labels in multi-photon imaging of cells and tissues (in collaboration with University Bonn, Ch. Thiele, P10).

Our research achievements demonstrate the benefits and high potential of label-free Raman and CARS microscopies as complementary and non-invasive tools for further compelling applications in lipidomic research that circumvent current limitations in fluorescent-based microscopy approaches, and provide

information that cannot be obtained by conventional biomedical microscopies and analytical techniques in a non-invasive manner. The advances made within this project are currently prepared for publications in peer-reviewed journals.

Work progress and achievements during the period for WP3

The most significant results from WP3 are the in depth analyses of several new pathways that are key players in altered LD formation and contribute massively to the etiology of obesity and diabetes (diabesity). Due to a collaborative effort we were able to identify the major components of some of these pathways also uncovering new druggable targets that might possibly be used for diabetes therapy. Furthermore, we could show by combing analyses from human and mouse data that considerable proportion of these pathways are conserved allowing us to use the mouse model to better understand the progression of human disease. The detailed analyses are listed in the report below.

Lipid / protein interaction affecting LD assembly and disassembly

For lipid-protein and protein-protein interaction studies Protagen's UNIclone expression libraries were screened for expression clones for specific lipid binding proteins. All expression clones exhibiting a lipid binding protein insert can be used for further lipid binding studies.

To combine the protein array technology with MS, initially proteins have been coated on nitrocellulose stripes, followed by incubation and washing steps. The bound protein was then extracted from the nitrocellulose matrix and identified by MS. However, the test quality obtained with nitrocellulose as matrix was not sensitive enough due to high background noise and large CV of 10 to 40 %. Additionally, when increasing the number of immobilised proteins, technical limitations such as spot finding were experienced. As an alternative, a bead-based protein array based on the Luminex technology was established for interaction studies. To set up such an assay, two different coupling procedures were developed with regard to proteins purified in two different buffer systems. One buffer system is compatible to physiological conditions, the other system is based on denatured conditions. Refolding of structural epitopes may occur directly on the bead during sample incubation.

This technology can be used in future studies not only for studying the interaction of lipids and proteins but also to study other molecules that might interact with different protein classes.

In a targeted approach we used a gene array screen to identify genes which are deregulated in obese and type 2 diabetic patients and mice (WP3 and WP4). The list was filtered for possible lipid interactors which led to the identification of the orphan transcription factor ROR-gamma as a key driver of adipocyte formation.

Through an MS coupled approach we were able to identify a tetra-hydroxylated bile acid as one endogenous ligand of ROR-gamma. We could demonstrate that dietary supplementation of THBA was able to protect from obesity associated type-2 diabetes development. Mice treated with THBA or CA showed no difference in weight gain over the course of six weeks. However, mice treated with THBA had significantly smaller adipocytes due to a massive increase in adipogenesis. As a consequence THBA

treated mice retained their insulin sensitivity and did not develop type 2 diabetes in comparison to CA and HFD treated mice. Taken together we identified a functional lipid-protein interaction which might be important for modulating adipose tissue response to energy overload. As THBA is a natural occurring lipid it can be used in dietary intervention to treat obesity associated insulin resistance.

Metabolic disorders and aberrant LD formation in the adipocyte

The objective of this part of WP3 was to establish pathways that are essential for aberrant LD formation in adipose tissue using animal models of metabolic disorders. Several new pathways were identified which might contribute to the etiology of type 2 diabetes development.

To identify key signalling nodes that regulate LD formation and degradation in the mature adipocyte transcriptomics data from seven different animal models of metabolic disorders was analysed in collaboration between several partners using the bioinformatics platform Explain. The workflow to identify keynodes that can be used to model the transcriptional changes can be has been for the first time applied to the analysis of metabolic disorders. From these differentially regulated genes promoter information was extracted and used to calculate a Transcription factor matrix. The matrix was used as a basis to identify key signalling nodes that regulate adipocyte function and that might be responsible for altered function in states of metabolic disorders.

Caveolins form plasmalemmal invaginated caveolae, and the total absence of caveolin-1 by global gene invalidation in mice results in defective adipose tissue lipid storage capacity leading to progressive lipoatrophy, and resistance to high fat feeding induced obesity. Since caveolin-1 is highly expressed in two different cell types namely adipocytes and endothelial cells, and a close interconnection with vascular network is of crucial importance for adipose tissue nutrient storage function, we have examined the question of whether defective lipid accumulation could be related to disrupted endothelial and/or adipocyte caveolin expression. To answer this question, we took advantage of a mice model with tissue-specific caveolin deficiency to dissect the respective roles of adipocyte and endothelial caveolin. Adipose tissue development was have analysed in Cav1 null mice with global invalidation for Cav1 (Cav1-KO) and in Cav1 null mice in which caveolin-1 expression was rescued exclusively in endothelial cells but not in adipocytes (Cav1-RC).

We could show that a lack of caveolin in adipocytes but not in endothelial cells accounts for adipose tissue lipoatrophy, decreased adipocyte size and adipocyte LD diameter reduction. In addition, we also observed that endothelial caveolin regulates macrophage infiltration and extravasation of immune cells into adipose tissue, thus unravelling distinct roles of endothelial and adipocyte caveolins. Indeed caveolin-deficient mice exhibit macrophage infiltration in their adipose tissue (as assessed by Mac2 and F4/80 immunohistology), which was totally rescued by caveolin re-expression in endothelial cells.

In addition we could show identify new pathways linking inflammation and especially immunoglobulins (Ig) released by B-lymphocytes as key drivers in the pathogenesis of diet-induced insulin resistance and type 2 diabetes. Recent studies have suggested that during the development of obesity Igs play a key role in this pathology, although their mechanism of action is still unknown. Ig activate cellular responses by cross linking membrane receptors specific for the Fc portion of the Ig molecule (FcR). We have investigated

whether functional FcR play a role in the development of diet-induced adipose tissue inflammation and diet-induced insulin resistance. To this end, FcR gamma-chain -/- mice that are charactersed by a decreased FcR-mediated activation by IgG and IgE antibodies, were fed a high fat diet (HFD). FcR gamma-chain-/- mice gained less weight on HFD which was caused by a decreased positive energy balance compared to wild-type (WT) controls. A decreased postprandial response to a lipid load was observed, which suggests altered lipid absorption in the intestine of FcR gamma-chain -/- mice. Basal glucose and insulin levels were decreased in FcR gamma-chain -/- mice compared to WT controls and hyperinsulinemic euglycemic clamp analysis revealed increased peripheral insulin sensitivity in FcR gamma-chain -/- mice. The adipose tissue of FcR gamma-chain -/- mice expressed lower levels of inflammatory genes and contained no crown-like structures. Taken together, these results demonstrate that the FcR gamma-chain acts as a sensor for HFD induced obesity leading to insulin resistance and adipose tissue inflammation. Adiponutrin, another novel target, (ADPN) belongs to the patatin like phospholipase domain containing protein family, annotated as PNPLA3, a close relative of PNPLA2 a.k.a. ATGL. Both proteins locate to the surface of LD and are believed to be lipases. Interestingly, in humans a non-synonymous genetic polymorphism in ADPN (I148M) has been correlated to fatty liver diseases, even up to cirrhoses, but underlying mechanism were not known. Here, we could demonstrate in enzymatic tests with membrane and LD fractions hat these proteins act as an LPAAT, specific for LPA and long-chain acyl-CoA. These data indicate that ADPN, though having a slow TAG hydrolase activity, is not involved in degrading lipid, rather in lipid synthesis. To this end, we engineered the I148M variants for both human and murine ADPN and observed when working with fractions from cell cultures a direct gain in function not only in LPAAT activity, but also an increase of TAG and PC contents as determined by incorporation of oleic acid originating from 14C-labelelled oleoyl-CoA. Of note, activities of the human proteins were always higher than those of the murine proteins. Based on the fact that high-sucrose diet (HSD) triggers enhanced expression of ADPN in murine liver thus leading to various stages of fatty liver diseases, we carried out a nutritional study with ADPN-WT and ADPN-KO mice fed either chow or HSD. No differences were found in animals fed chow, but upon administration of HSD the ADPN-KO mouse revealed a strong phenotype at the levels of liver homogenates and LD, by having reduced LPAAT activities as well as reduced lipid stores. Overall, ADPN is involved in lipogenic paths by its LPAAT activity that is even enhanced by the I148M variant leading to liver pathologies. These date provide evidence that this mouse model is excellently suited for elucidation of the human situation. Based on the data from functional analysis of adipocyte transcriptomics as well as from different animal models it became clear that one major hub for the regulation of LD formation and adipogenesis is extracellular matrix reorganisation which is a crucial step of adipocyte differentiation and is controlled by the MMP / TIMP enzyme system. One pertinent regulator of this process is the protein TIMP1.

TIMP1 mRNA and protein levels were measured in lean and obese animals also we analysed human samples for circulating Timp1 levels as part of the translational analyses. TIMP1 expression is increased in the serum and adipose tissue of obese mouse models. Recombinant murine TIMP1 is an inhibitor of adipocyte differentiation in subcutaneous primary preadipocytes. This is of special interest since it is possible that altered TIMP1 levels in visceral adipose tissue might explain the increased contribution of this fat depot to the development of metabolic disorders.

In vivo, injection of recombinant TIMP1 in mice challenged with a HFD leads to enlarged adipocytes. TIMP1-treated mice develop an impaired metabolic profile with increased circulating free fatty acid (FFA)

levels, hepatic triacylglycerol accumulation and accelerated insulin resistance. Altered glucose clearance in TIMP1-injected mice is due to changes in adipose tissue glucose uptake, whereas muscle glucose clearance remains unaffected. In conclusion it can be stated that the keynode TIMP1 identified by HTP screening as reported above is important for mouse and human physiology is a negative regulator of adipogenesis. In vivo, TIMP1 leads to enlarged adipocytes in the state of over-nutrition. This might contribute to the detrimental metabolic consequences, such as systemic fatty acid overload, hepatic lipid accumulation and insulin resistance.

Metabolic disorders and aberrant LD formation in the hepatocyte

The objective of this part of the WP was to establish pathways that are essential for aberrant LD formation in hepatocytes and hepatic stellate cells (HSCs) using animal models of metabolic disorders.

It is interesting to note that nutritional stress exerted by starvation or HFD and genetic stress exerted by malfunctioning adipose triglyceride lipase (ATGL) always result in various forms of non-alcoholic fatty liver diseases (NAFLD), the latter known in humans as 'neutral lipid storage disease with cardiomyopathy'. We postulated that the different etiologies for the steatoses observed in mouse models should have an impact on the lipidome of respective hepatocyte LD. The experimental conduct to test our working hypothesis consisted, of several intervention studies with male C57BL/6 wild-type (WT) and ATGL knock-out (KO) mice that received for 6 weeks control chow (FED), HFD, or were fasted for 16 h before sacrifice (FAS). From excised livers hepatocytes and there from LD were isolated, lipids extracted and subjected to lipidomic analysis. Lipidclass-specific analyses were carried out at levels of lipid species (profiling) and lipid molecular species. Samples analysed were from mouse groups WT-HFD, WT-FED (control), WT-FAS, KO-FED and KO-FAS (super stress). First, we could show that LD numbers in freshly isolated hepatocytes were strongly induced upon HFD or starvation. The KO-effect is not as dramatic and notably the combined genetic and nutritional stresses ('super stress', KO-FAS) reversed these pathogenic phenotypes almost to a phenocopy of the control (WT-FED). With over 97 mol% the TAG class in hepatocyte LD was the prominent one among minor DAG, PC, LPC, PE, PS, PI and SM classes, where some significant differences in class composition due to nutritional and genetic stresses were found.

Lipidomic profiling revealed that the TAG class responded most sensibly to stresses applied to the animals. In all experimental groups examined we identified after LC-MS around 110 TAG species with acyl chain-lengths ranging from C28 to C62 having 0 to 15 double bonds. In the profiles C50 to C58 species always predominated with amounts between 1 to 23 % relative to total amount TG species, abundance of those having lower and higher chain-lengths were below the 1 % line. Actually, the number of TAG molecular species determined by MS/MS was much higher due to multiple variations of acyl-chains fitting one TAG species. In the first set of intervention studies comparing WT-HFD, WT-FED and WT-FAS and in the second set comparing WT-FED, KO-FED, WT-FAS and KO-FAS principle component analyses (PCA) of TAG species profiles was able to separate the data into the 3 and 4 groups, accordingly. In the second set, for example, we found that the FAS effect triggered in principle component 1 (PC1) a shift of TG species to the left, the KO-effect to the right and, most interestingly, super stress almost ?neutralised? individual nutritional and genetic stresses in PC1. Taken together, TG species and molecular species in hepatocyte LD provide the lipidome best suited to phenotyping and diagnosing steatoses of various etiologies. Moreover, quantitative determination and structural analysis of acylglycerol and phospholipid

species furnish metabolic insights.

HSC, the non-parenchymal cells located peri-sinusoidally in the Space of Disse, represent 5-8 % of the total liver cell population. Under physiological conditions, HSC have been defined as the most important reservoir for vitamin A in mammals, stored in a large number of cytoplasmic LDs containing cholesteryl esters (CE), triacylglycerols (TAG) and retinyl esters (RE), the storage form of vitamin A. Activation of HSCs has been recognised as one of the first steps in liver injury and repair. During activation, HSCs transform into myofibroblasts with concomitant loss of their LD and production of excessive extracellular matrix. Changes in the metabolism of retinoids and release of the hydrophobic lipid content associated with transformation of LDs are considered to be one of the main factors connected with HSC activation. We have analysed different animal models based on our findings from transcriptomic analyses established in year 1-2 of the project.

The molecular identity of the enzymes involved in LD degradation in HSCs during activation is largely unexplored. We therefore studied the LD breakdown in HSCs isolated from mice lacking adipose triglyceride lipase (ATGL), an enzyme known to have an important role in LD degradation in many eukaryotic cells. We found a similar neutral lipid profile in freshly isolated HSC from ATGL-WT and ATGL-KO mice, including similar levels of multiple TAG, CE and RE species. Like in wild-type HSC, the neutral lipids were broken down for 80-90 % in ATGL-deficient HSC after 6 days in culture, suggesting involvement of other lipases than ATGL in LD breakdown in HSC. Microarray analysis showed that ATGL is expressed in freshly isolated, quiescent, HSC and in culture-activated HSC to the same extent. However, there was no difference in the induction of genes during activation between HSC from ATGL-WT and ATGL-KO mice, indicating that ATGL deficiency does not affect activation of HSC. A main feature of HSC is the storage of RE in large LD. The enzyme Lecithin retinol acyltransferase (LRAT) is responsible for esterifying more than 90 % of this retinol within the cells. Surprisingly, previous studies on LRAT knockout mice show dramatic decreases of RE levels in different tissues and a complete absence of HSC LD when compared to wild type mice. We now show that HSC from LRAT knockout mice cultured under several conditions were able to form RE into LDs. RE formation in HSCs showed different kinetics in wild-type and LRAT knockout mice when measured by means of HPLC-MS / MS. As a consequence, an acy-CoA:retinol acyl transferase (ARAT) activity is likely to be responsible for RE formation in HSC from LRAT knockout mice. These results also suggest that the absence of LD in HSC from LRAT knockout mice may be due to the impaired absorption of retinoids by the intestine and not due to the lack of LRAT activity.

Regulation of the hepatic LD formation

The search for lipidomic biomarkers in the EUROSPAN genome-wide association study (GWAS) combines single nucleotide polymorphisms (SNPs) with lipid species data, disease risk and morbidity. Based on this and our own LIPIDOMICNET study (WPs 2 to 4) we identified polymorphic genes in sphingolipid / fatty acid metabolism that are significantly associated with circulating lipid species and diabesity. In fact, SNPs rs10938494 and rs2351791 in the ATP10D gene were significantly associated with circulating C16:0 and C24:1 glucosylceramide species levels, obesity and insulin resistance. ATP10D is a P4 ATPase associated with HDL remodelling in mice that may be involved in lipid transfer from either the exoplasmic or endoplasmatic reticulum/Golgi luminal side to the cytoplasmic membrane leaflet. C57BL/6 mice express a truncated form of ATP10D (caused by the SNPs referred to above) and easily

develop obesity and insulin resistance on high-fat diet (HFD). Based on these findings, we analysed the metabolic function of ATP10D in Atp10d deficient (Def) and transgenic (TG) C57BL/6 mice. Compared to ATP10D TG mice, Def mice gain 20 % more weight on HFD and revealed significantly increased triglyceride (TAG) levels and decreased oxygen consumption/CO2 production. Furthermore, significantly elevated glucose and insulin levels and partially reduced insulin sensitivity were visible in Def mice. Def mice show also elevated hexosylceramide species levels. Transcriptomic analysis indicates that ATP10D affects the peroxisome proliferator-activated receptor (PPAR)-gamma regulatory network. Analysis of the hepatic expression profile of ATP10D transgenic and deficient mice indicated involvement of a transcriptional network of lipid and/or glucose metabolism associated genes containing PPAR-gamma, PPAR-alpha and HNF4-alpha. Transcription of Cidec was described to be regulated by CCAAT / enhancer binding protein (C/EBP) and PPAR-gamma. CIDEC (FSP27) together with CIDEA and CIDEB belongs to the cell death-inducing DFF45-like effector (CIDE) family. Recently this family was found to regulate energy homeostasis. In adipocytes CIDEC is localised on LDs. Cidec-KO mice are protected from diet-induced obesity, show elevated glucose uptake rates and better insulin sensitivity, and increased lipolysis rate. They also display decreased TAG levels in white adipose tissue (WAT). WAT of Cidec-KO mice acquires brown adipose tissue (BAT) features.

Stearoyl-CoA-desaturase-1 (SCD1) is known to be a keynode enzyme for lipid and glucose metabolism. Interestingly, SCD-1 expression was found to be increased in Def mice, both on mRNA (approx. 4 fold) and protein level. The SCD-1/Actin ratio was approx. 3 fold higher in Def mice than in TG mice. Along with altered SCD1 expression FA and lipid species patterns were changed. There was a significant increase in the 18:1 / 18:0 free FA ratio of Def mice compared to TG mice. Similar 18:1/18:0 and 16:1/16:0 shifts were observed in LPC and CE. These findings demonstrate that the observed elevated expression of SCD1 has functional relevance and leads to enhanced desaturation of its substrates. A defect in the ATP10D gene leads in liver, in response to HFD, to enhanced obesity and insulin resistance in liver and plama, as well as enhanced keynode SCD-1 expression in liver, the latter verified also by enhanced 18:1/18:0 ratios. As shown also in this study, these effects become ameliorated by the repaired gene in the transgenic ATP10D mice.

Potential impact:

Metabolic overload is a paramount problem for people in industrial countries due to associated risks of secondary disorders including obesity, diabetes, atherosclerosis and hypertension, with obesity being the entry gate for type 2 diabetes (T2D), and it is estimated that more than 50% of the population in developed countries can be regarded as obese according to the standards of the WHO. Moreover, obesity is the entry gate for T2D. Thus, it is not surprising that pandemics of obesity will be soon followed by an epidemic of diabetes. By 2020 it is estimated that 130 million people will be afflicted by T2D diabetes with an estimated cost of USD 95 billion for health care.

EU-FP7 LIPIDOMICNET (see http://www.LIPIDOMICNET.org online) project aimed to tackle the problems of metabolic overload strengthening lipidomics research in Europe. Uniting academic and industrial members, 25 partners (20 academic research groups and 5 small and medium-sized enterprises (SMEs)) jointed their forces investigating lipid storage and release. The technical advances, HT technologies, provided the optimal methodological possibilities to map the entire spectrum of lipids in cells,

tissues and whole organisms.

The recent developments in lipid MS have set the scene for a completely new way to understand the composition of membranes, cells and tissues in space and time by allowing the precise identification and quantification of alterations of the total lipid profile after specific perturbations. In combination with advanced proteome and transcriptome analysis tools and novel imaging techniques using RNA interference, researchers of LIPIDOMICNET project could unravel the complex network between lipids, genes and proteins in an integrated lipidomics approach. Translational research from mouse to man applied to LD pathology was another cornerstone of this large-scale project interfacing research and development.

LIPIDOMICNET was built on a private public partnership to support the translation of LIPIDOMICNET inventions into new technologies and products that will benefit the health care systems. The 5 SMEs BIOBASE (see http://www.biobase.de online), Institute of Systems Biology (ISB) (see http://www.systemsbiology.ru online), ZORA Biosciences (see http://www.zora.fi online), Integromics (see http://www.integromics.com online) and Protagen (see http://www.protagen.de online) were the industrial partners to proof once more that optimal merging of academic and industrial interests can be a reliable basis for successful cooperation, comprising the core competence of such a consortiums as LIPIDOMICNET.

The value of the assembled data generated throughout the project, is being organised as a detailed special purpose Wiki base available at http://www.lipidomicnet.org. LIPIDOMICNET was linked and collaborated with the NIH initiative LIPID MAPS (www.lipidmaps.org) and the Japanese pendant Lipidbank (see http://www.lipidbank.jp online) as well as other European initiatives such as European Network for Oxysterol Research (ENOR) (see http://oxysterols.com/ online). It is closely connected with the Danubian Biobank consortium (SSA DanuBiobank, see http://www.danubianbiobank.de online) for clinical lipidomics, DEEP (see http://deutsches-epigenom-programm.de online, funded by BMBF, Germany) for epigenetics, SysMBo- for systems Biology (funded by BMBF, Germany) as well as to other local national projects of a smaller scale. LIPIDOMICNET consortium promoted lipidomic research and development attracted the best young investigators to this newly forming research field to safeguard Europe's vital interests in this important area, and to ensure successful cooperation with the United States of America (USA) and Asia. Funding LIPIDOMICNET and thus promoting the field of Lipidomics was of benefit for areas such as health, nutrition and disease management.

List of websites: http://www.LIPIDOMICNET.org
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Documenti correlati

Final Report - LIPIDOMICNET (Lipid droplets as dynamic organelles of fat deposition and release: translational research towards human disease)

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