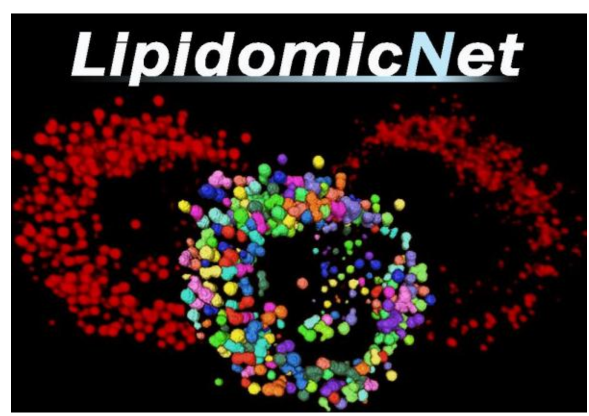

PROJECT FINAL REPORT

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4.1 Final publishable summary report

An executive summary

The consortium invested major efforts to develop new high-throughput 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 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, esp. 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 characterized 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-signaling in atherosclerotic lesions, the impact of lipids on monocyte/macrophage differentiation was studied. Novel methods for isolation and multi-omic characterization of cells, extracellular vesicles 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 characterize 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 characterized. The LipidomicNet wiki (http://www.lipidomicnet.org) was made public and all standard processing procedures 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, miRNA and proteomic data.

A summary description of project context and objectives

The LipidomicNet project aimed to exploit the recent developments in lipidomics to establish high-throughput 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 program, the following major scientific and technological objectives of this highly focused S&T project were set:

- To develop high-throughput 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.
- To 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 characterize 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 endolysosomes (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 synergize knowledge and data analysis generated by lipidomic, proteomic, genomic, and transcriptomic high-throughput (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) aimed to achieve successful management of the consortium activities. Management work-package members supported the beneficiaries through all reporting periods mediating optimal communication between 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) 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 mass spectrometry, proteomics, transcriptomics and others were embedded into various subgroups. As a horizontal communication structure, task forces were established that organized the contact between groups working on related problems. All technologies, both newly developed and improved or standardized, 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 lipid droplet processing) 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 WP 2 under the aspect of development of high-throughput MS technology for lipidomic analysis and, by the same token, with WP7 for data integration and deposition, as well as their interpretation by bioinformatics 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 lipid droplet processing in human diseases) was directed towards determining pathways regulating lipid droplet 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 invitro 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 maximized. By adopting an 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 lipid droplet-regulating signaling pathways aimed to extend our knowledge of cellular lipid and protein kinases and phosphatases regulating influx, efflux and storage in lipid droplets 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 lipid droplet formation and HCV infection.

WP5 (Lipid droplets and lipid bodies in human macrophages and leukocytes) 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 analyzed for their functional properties and evaluated for their use as clinical biomarkers. Using bioinformatics correlation analysis, the underlying regulatory networks were analyzed. 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 eLDL and oxLDL and the cellular lipid storage pattern visualized by fluorescence microscopy as well as performing lipidomic and transcriptomic analysis. Cells from Niemann-Pick type C disease patients were used to further characterize 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 apoA-I were studied in vitro in monocyte-derived-macrophages. The oxLDL induced lipid droplet 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 signaling. 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. Extracellular vesicles from granulocytes were characterized in their lipidomic, miRNA and proteomic properties and functional aspects in atherosclerotic plaque maintenance studied. Extracellular vesicles (EV) 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 Lipid Droplets and Lamellar Bodies) was related to cellular lipid entry pathways into endolysosomal lamellar bodies and lipid droplets. 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- (Niemann Pick disease, type C-) 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 characterization of lipotoxic drugs and their role in lipid droplet formation has been addressed with the aid of lipid probes that have been modified by fluorescence, isotopes, and other structural modifications. Mass spectrometry has been applied for the characterization of lipid uptake pathways, and its application to lipidomics has been extended.

WP7 (Bioinformatics) aimed at maximizing 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 standard processing procedures (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 mass spectrometry (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-labeled and stable isotope labeled lipid species (analyzed by Mass Spec) was developed, including features such as the visualization 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)

Enhanced communication tools being continuously optimized 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, organizing 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 work-package 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. Among 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.

A description of the main S&T results/foregrounds

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 mass spectrometry 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 lipid droplets (LD) 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 high-throughput methods to define druggable 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 work-packages (2-7) 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 lipid droplet 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 platelet lipid droplet dye with improved specificity and stability.** LD540 is a lipophilic dye, based on the Bodipy fluorophore, for microscopic imaging of lipid droplets. In contrast to previous lipid droplet dyes, it can be resolved spectrally from both green and red fluorophores allowing multi-color 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-color imaging lipid droplet 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 (enzymatically degraded LDL) and Ox-LDL (oxidized LDL), into human macrophages. In summary, this study has demonstrated quantitative hyperspectral CARS imaging for the noninvasive and label-free study of Ox-LDL and E-LDL induced lipid droplets 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 lipid droplets, in **EM-tomographic analysis of lipid droplets and in EM lipid tracing using click chemistry.** These methods are particularly useful to study the complex organization and the rapid dynamics of hepatic lipid metabolism and have direct impact on the work in other work-packages, in particular WP5 and WP6.

Using **freeze fracture electron microscopy**, possible pathways of lipid droplet growth were studied. Homotypic fusion of small lipid droplets 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 lipid droplets 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 lipid droplets frequently appear to be aggregates of smaller droplets. The overwhelming impression created by these images is that numerous smaller lipid droplets have amalgamated to create a larger one. One of the theoretical objections to fusion as a mechanism of lipid droplet 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.

Mass spectrometry

Large amounts of different methods for mass spectrometrical lipid analysis were either newly developed or were improved and standardized or automated high throughput analysis. For **bile acid species quantification,** a simple, sensitive liquid chromatography-tandem mass spectrometry (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% 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 oxyphytosterols 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, Niemann-Pick Type C 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 analyze >700 molecular species in a single run. The system has been characterized 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 mass spectrometry. 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 mass spectrometry.** There has been a long standing difficulty with determining the phosphoinositides using mass spectrometry (MS). There are a number of reasons for this: the phosphoinositides are signaling molecules, consequently there generation and removal is tightly regulated and the more highly phosphorylated forms particularly PtdIns3,4,5P_3 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 stabilizes 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 PtdIns3,4,5P_3 species, a similar approach can identify PtdInsP_2

though it is not possible at this stage to fully characterize PtdIns4,5P_2 from PtdIns3,4P_2 or PtdIns3,5P_2. 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 noninvasive, label-free, and quantitative *in-situ* chemical analysis of lipid species with high spatial resolution (sub-femtoliter 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 noninvasive identification of 7-Ketocholesterol as one of the lipid oxidation products in atherogenic oxidized lipoproteins (in collaboration with University Hospital Regensburg, G. Schmitz, P01);

A. The noninvasive chemical mapping of lipid droplet composition in living human macrophages (in collaboration with University Hospital Regensburg, G. Schmitz, P01);

B. The Raman-spectroscopic characterization 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 noninvasive 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 noninvasive 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 lipid droplet 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 lipid droplet 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 mass spectrometry, 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 mass spectrometry. However, the test quality obtained with nitrocellulose as matrix was not sensitive enough due to high background noise and large coefficients of variation (CV) of 10->40%. Additionally, when increasing the number of immobilized 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 RORγ 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 RORγ. 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 lipid droplet formation in the adipocyte

The objective of this part of WP3 was to establish pathways that are essential for aberrant lipid droplet 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 signaling nodes that regulate lipid droplet formation and degradation in the mature adipocyte transcriptomics data from seven different animal models of metabolic disorders was analyzed 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 signaling 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 analyzed 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 lipid droplet diameter reduction. In addition, we also observed that endothelial caveolin regulates macrophage infiltration and extravasation of immune cells into adipose tissue, thus unraveling 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 activates 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 γ-chain$^{-/-}$ mice that are characterized by a decreased FcR-mediated activation by IgG and IgE antibodies, were fed a high fat diet (HFD). FcR γ-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 γ-chain$^{-/-}$ mice. Basal glucose and insulin levels were decreased in FcR γ-chain$^{-/-}$ mice

compared to WT controls and hyperinsulinemic euglycemic clamp analysis revealed increased peripheral insulin sensitivity in FcR γ-chain$^{-/-}$ mice. The adipose tissue of FcR γ-chain$^{-/-}$ mice expressed lower levels of inflammatory genes and contained no crown-like structures. Taken together, these results demonstrate that the FcR γ-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 ^{14}C-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 lipid droplet formation and adipogenesis is extracellular matrix reorganization 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 analyzed 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 high fat diet leads to enlarged adipocytes. TIMP1-treated mice develop an impaired metabolic profile with increased circulating free fatty acid levels (FFA), 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 lipid droplet formation in the hepatocyte

The objective of this part of the work package was to establish pathways that are essential for aberrant lipid droplet formation in hepatocytes and hepatic stellate cells (HSC) using animal models of metabolic disorders.

It is interesting to note that nutritional stress exerted by starvation or high fat diet 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 steatosis 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), high fat diet (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 analyzed 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 C_{28} to C_{62} having 0 to 15 double bonds. In the profiles C_{50} to C_{58} 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 "neutralized" 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 steatosis 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 lipid droplets containing cholesteryl esters (CE), triacylglycerols (TAG) and

retinyl esters (RE), the storage form of vitamin A. Activation of hepatic stellate cells has been recognized as one of the first steps in liver injury and repair. During activation, hepatic stellate cells 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 lipid droplets are considered to be one of the main factors connected with HSC activation. We have analyzed 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 lipid droplets. 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 lipid droplet formation

The search for lipidomic biomarkers in the EUROSPAN GWAS combines SNPs with lipid species data, disease risk and morbidity. Based on this and our own LipidomicNet study (WP2, 3 and 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 analyzed 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 PPARγ 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 PPARγ, PPARα and HNF4α. Transcription of Cidec was described to be regulated by CCAAT/enhancer binding protein (C/EBP) and PPARγ. 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 localized on lipid droplets. 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 plasma, 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.

Work progress and achievements during the period for WP4

WP4 aimed to study the factors regulating lipid droplet formation in human cells and tissues with a major focus upon liver and adipose tissue. This information was used in parallel with studies upon lipid-associated diseases in particular diabetes and steatosis together with pathologies associated with for example hepatitis C virus (HCV) infection with the aim of highlighting potential therapeutic targets. This work benefited from the study both of human cells and model cell lines. In addition there was a close association with and collaboration with WP3 so that data generated in mouse model systems could both inform and benefit from studies in mouse models. Finally, a critical approach adopted by WP4 was the integration of top down and bottom up studies making use of human cohort studies. Practically the WP included groups with expertise in lipidomics, proteomics and transcriptomics and benefited from the input of the bioinformatics groups in WP7.

1. Identification of genes involved in lipid droplet processing

In order to identify critical genes an automated siRNA screen was developed which made use of the LD540 lipid dye developed by Thiele (Spandl et al., 2009). For a first screen, the Ambion kinase and phosphatase siRNA library was used, which contained about 3900 siRNAs against 1280 genes. After an initial three rounds of screening, two additional rounds of screening were performed to broaden the basis for the following multiparametric statistical analysis of the imaging data. A first set of 76 hits that were identified in this procedure were re-screened using (i) the original siRNA sequences, (ii) 364 additional siRNA sequences from two different vendors and (iii) 54 esiRNA sequences generated by RNAse III digestion of longer double stranded RNAs, resulting in a coverage of 6-9 sequences per gene. 14 genes scored in at least 40 % of these sequences and were included into a final hit list. A number of gene products have been identified through this approach. The most important gene identified is AUP1 – a new protein with interesting functions including ubiquitination and a role in droplet clustering.

2. Regulation of lipid droplet formation

In parallel with the analysis of key processes by siRNA screening the importance of particular signaling pathways in controlling lipid droplet formation was studied using the Huh7 hepatocyte cell line. These studies pointed to a key role for the PI3kinase and phospholipase D pathways, but no role for the MAP kinase pathway which had been suggested previously. Additionally the importance of long chain fatty acid receptors, in this case GPR120 though in other situations GPR40, in controlling lipid droplet formation in response to increased exposure to fatty acids (manuscript submitted to J Lipid Research). This work highlights the potential for relevant inhibitors of these pathways in preventing droplet formation. Studies which showed an increase in obesity in patients with Cowden’s disease, where the PI3kinase pathway is amplified (Pai et al., 2012), support this proposal.

Single molecule tracing microscopy was used to characterize the involvement of lipid microdomains in lipid droplet formation. This work also pointed to an important role for heat shock proteins in lipid droplet formation. Fever-like heat stress or heat-analogous mild membrane stress was found to promote triglyceride synthesis and thus droplet formation. Understanding the pathways involved which are regulated by HSF-1 again points to potential therapeutic targets.

3. Target identification in disease states

HCV infection is promoted by virus replication on lipid droplets. Thus WP4 utilized lipidomic analysis to determine changes in lipid droplet composition in control and virus infected cells and determined the effects of inhibitors of triglyceride synthesis upon HCV infection. The data demonstrates that sub-toxic doses of Triacsin C (an inhibitor of long fatty acyl CoA synthetase) depletes cellular cholesterol ester and thus lipid droplets whilst YIC-C8-434 (an acylCoA:cholesterol acyltransferase inhibitor) induces larger lipid storage structures and affected acyl chain structures particularly double bond number. Both inhibitors reduced replication of the viral genome. In a parallel study changes in gene expression was examined in patient cohorts that were HCV positive or negative; this cohort were infected with the gt1a strain. The transcriptomic analysis has identified a cluster of just over 30 genes that had higher expression in 8 out of 9 biopsies from HCV-infected patients. These genes were primarily involved in the interferon response pathway. There was no evidence for differential regulation of genes involved in lipid metabolism. Ongoing studies are examining biopsies from patients infected with HCV gt3a strains, which are more frequently linked to metabolic changes in the liver that result in steatosis.

The top down approach utilized the patient cohorts in the AIDA/ Acarbose Type 2 Diabetes Study, Ludwigshafen Risk and Cardiovascular health (LURIC) and EUROSPAN studies as well as additional cohorts from weight loss, oral fat load and obese prediabetic and diabetic patient studies. The studies included both lipidomic and transcriptomic analysis. This has identified significantly elevated autoantibody levels directed against 26 proteins associated to LD-droplet processing and vesicular trafficking upon weight loss. Additionally WP4 have identified genes showing significant associations with sphingolipid species and those showing significant associations with circulating phospholipid species that are genetic risk factors for complex diseases. This approach revealed SNPs in the ATP10D gene showing significant association with circulating C16:0 and C24:1 glucosylceramide species and vascular and metabolic disease. In the LURIC study saturated and monounsaturated phosphatidylcholine species, especially PC 32:0 (most probably dipalmitoyl-phosphatidylcholine), were found positively associated with total and cardiovascular mortality while highly polyunsaturated phosphatidylcholine species supposed to contain n-3 fatty acids similar to lyso-phosphatidylcholine species (16:0, 18:0, 18:2) indicated survival. Sphingomyelin 16:0, 16:1, 24:1 and 24:2 species showed positive association with mortality. Similar tendencies were observed for the ceramides 16:0, 18:0 and 24:1. In contrast sphingomyelin 22:0, 23:0, 24:0, 22:1 and 23:1 together with ceramide 23:0 and 24:0 showed a negative correlation with mortality. Interestingly sphingomyelin and ceramide 24:0 were protective, while their mono-desaturation products sphingomyelin and ceramide 24:1 showed positive association with mortality. GWA identified among others the fatty acid desaturases FADS1 and FADS2 and SIRT3 and TMEM135, both involved in mitochondrial energy metabolism. Candidate genes including ATP10D, FADS1/2/3 and ELOVL2 and lipid species such as C16:0/18:0/24:1 were identified. To understand the molecular mechanism of ATP10D an integrative project was established between WP3 and WP4 using in vitro cell experiments, mouse models and additional patient material.

3. Lipids in adipocyte differentiation

During adipocyte differentiation significant upregulation of stearoyl-CoA desaturase (SCD1 = FADS5) was observed. SCD1 catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids. The principal product of SCD is oleic acid, which is formed by desaturation of stearic acid. Increased expression of SCD1 leads to increased levels of mono-unsaturated fatty acids (MUFAs) and decreased levels of poly unsaturated fatty acids (PUFAs) whereas no change in saturated fatty acid levels (SAFAs) could be detected. Oleate was found elsewhere in WP4 to be the major fatty acid capable of promoting lipid droplet formation.

Human adipose tissue contains a population of non-characterized cells that are able to undergo adipogenic, osteogenic, chondrogenic or miogenic differentiation *in vitro* as well as *in vivo*. Clonally analysis of such cells, called stromal-vascular fraction (SVF) cells, demonstrated, that they constitute a mixed population of cells that possess potential of differentiating into adipocytes or endothelial cells. In the study Human SVF have been isolated from subcutaneous fat tissue during surgery from healthy young woman (30-40yrs) with no metabolic syndrome and differentiated in culture.

Accumulation of TG was determined during the differentiation process (maximal amount was detected after 15 days of differentiation especially after incubation with PA and OA), while during the dedifferentiation process the amount of triglycerides significantly decreased the strongest effect was observed after incubation with EPA in presence of FCS or L-arginine. The energy state of the differentiating human subcutaneous adipose SVF progenitor cells and endothelial cells and changes in gene expression, number of lipid droplets in cells in "metabolically overload" conditions was examined in the presence of exogenous selected dietary saturated and unsaturated fatty acids. The data suggest that the possible formation of microparticles by differentiating SVF and their usage for extra- cellular signal transmission (Mause 2010, Baron 2011; Mueller 2011). These point to novel mechanisms of intercellular communication regulating lipid droplet generation that could be therapeutically tractable.

4. Cross talk between subcutaneous and visceral adipose tissue, alterations in adipose tissue during development of obesity and insulin resistance

Patient cohort studies have also identified the adipocyte expressed genes associated with obesity phenotypes such as % of body fat, BMI, waist circumference, adipocyte diameter which are in parallel associated with the free-living ambulatory activity. We have conducted fatty acid profiling of human subcutaneous and gluteofemoral triacylglycerol with the hypothesis that any difference between the tissues should depend on intrinsic metabolic processes such as de novo lipogenesis or desaturation elongation of fatty acids. Gluteofemoral tissues showed a very significant increase in 16:1w7, which has been postulated as an insulin sensitizing lipokine, whereas abdominal tissue showed signs of greater elongation (Pinnick et al., 2012). Based on these findings we have postulated that the metabolically advantageous properties of an expanded gluteofemoral fat depot, as seen in epidemiological studies, could, at least in part, be due to release of 16:1. The proportion of 16:1 in the NEFA fraction was strongly correlated with global insulin sensitivity. These studies further point to key therapeutic targets.

Transcriptomic analyses of human subcutaneous (SCfat) and visceral (VISfat) fat were performed to elaborate depot-specific characteristics in the ex vivo study from the same donor. This identified 525 genes showing lower expression in SCfat than in VISfat and 108

genes showing higher expression in SCfat, both in females and males. Classification according Biological Processes (GO) revealed that genes with lower expression in SCfat mainly are inflammation associated while genes with higher expression in SCfat are mainly development associated.

Work progress and achievements during the period for WP5

WP5 showed that during human monocyte to macrophage differentiation the major cellular lipid classes switched from cholesterol in monocytes (SREBP2 dominated) to monounsaturated fatty acid-enriched phosphatidylcholines in macrophages (SREBP1 dominated). Suppression of SREBP-1c activity and inhibition of fatty acid synthesis prevented monocyte differentiation and phagocytic macrophage function. Activation of SREBP-1c and subsequent induction of fatty acid and glycerophospholipid metabolism were essential for phagocytic differentiation of monocytes.

Dissection of oxidized LDL (oxLDL) and enzymatically modified LDL (eLDL)-induced cellular phenotypes indicated that these two atherogenic LDL-modifications were coupled with two fundamentally different cellular lipid storage responses in macrophages. Oxidized LDL preferentially up-regulated scavenger receptors, required for their internalization, induced lipid storage in the acidic compartment resembling drug-induced endolysosomal phospholipidosis, parallel with increased cellular content of the endolysosomal signature lipid bis(monoacylglycero)phosphate (BMP), pro-apoptotic signaling and appearance of ceramide-enriched surface membrane microdomains. By contrast, challenge of macrophages by eLDL led to expanded cholesterol- and sphingomyelin-enriched surface membrane microdomains, up-regulation of diverse pattern recognition receptors required for phagocytosis of eLDL, parallel with extensive lipid droplet formation, increased ER-stress and membrane contact site formation for interorganelle trafficking and signaling, and enhanced cellular content of the mitochondrial lipid cardiolipin (CL).

Cytoplasmic lipid droplets are formed as small primordial droplets at the ER-membrane and increase in size by a multistep fusion process. The fusion is catalyzed by the SNARE proteins SNAP23, syntaxin-5 and VAMP4. SNAP23 is involved in the insulin dependent translocation of GLUT4 to the plasma membrane, and has an important role in the development of insulin resistance. Thus FAs re-localize SNAP23 from the plasma membrane (and the translocation of GLUT4) to the cell interior giving rise to insulin resistance. This re-localization could be detected in skeletal muscles biopsies from patients with T2D compared to matched control, thus a missorting of SNAP23 is essential for the development of insulin resistance.

The role of plasmalogens and sphingolipids in monocyte to macrophage differentiation was investigated in our monocyte/macrophage in vitro differentiation model. For this purpose primary human monocytes were isolated by counterflow elutriation and differentiated by application of rhM-CSF. At different time points lipidomic, transcriptomic and miRNA analysis was performed monocyte to macrophage differentiation went along with a strong induction of fatty acid desaturation and elongation. The PE plasmalogen pattern on the other hand showed decreased PUFA and increased MUFA species becoming more similar to mature granulocytes. PE plasmalogens therefore might represent a potential biomarker for cell activation. An *in silico* network and transcription factor analysis revealed that this regulation is exerted only partly at the level of transcription, but is also involving miRNAs and targeting complex gene networks. Macrophages, in response to eLDL or oxLDL, also differentially altered their sphingolipid and cholesterylester profile. Lipidomic analysis revealed a significant lipid species shift, indicating a lipoprotein dependent adaption of the plasma membrane composition. The lipid species shift likely affected membrane microdomain formation and function, and is mediated at least in part by an altered gene expression profile. Activation of the salvage pathway (SMPD1&2, glucosidases) contributed to the cellular

response regulating differential lipoprotein uptake via phagocytosis (eLDL) or scavenger receptor uptake (oxLDL).

WP5 also showed that hypoxia/ischemia-induced accumulation of lipids in cardiomyocytes depends on expression of the VLDL receptor (VLDLR). Hypoxia-induced VLDLR expression in cardiomyocytes is dependent on HIF-1α through its interaction with a hypoxia-responsive element in the VLDLR promoter, and VLDLR promotes the endocytosis of lipoproteins. Furthermore, VLDLR expression is higher in ischemic compared with non-ischemic human tissue areas, and correlates with the total LD area in cardiomyocytes. Importantly, Vldlr-deficient mice show improved survival and decreased infarct area following an experimentally induced myocardial infarction. Ischemia-induced endoplasmic reticulum (ER)-stress and apoptosis are reduced in VLDLR-deficient mice, and in wildtype mice treated with antibodies specific for VLDLR. These findings suggest that VLDLR-induced lipid accumulation in the ischemic tissue worsens survival by increasing ER-stress and apoptosis.

In contrast to macrophages granulocytes undergo rapid apoptosis in response to eLDL-load. WP5 could show that this lipid stress leads to an extensive shedding of granulocyte-derived extracellular vesicles (EVs). These EVs have been implicated in a variety of signaling events. WP5 demonstrated that granulocyte-derived EVs chemotactically attract blood monocytes and convey an immunomodulatory effect on macrophages. This process is likely mediated by specific proteins of EVs, including annexins and integrins and specific phospholipids as ligands for G-protein receptors (GPRS) that modulate chemotaxis, migration and proliferation. Clinically this process could be involved in the sustained low grade inflammation in atherosclerosis.

Work progress and achievements during the period for WP6

Within WP6, lipid entry pathways have been studied with a focus on cultured cells. For this purpose, suitable tools have been developed by the participating groups. These are e.g. methods for the analysis of cellular lipid uptake and processing, the expression and purification of lipid transfer proteins, and the development of assay systems to analyze their function. Lipid probes labeled with isotopes, fluorescence or other reporter groups have been prepared and characterized, and cells with defined defects in proteins required for lipid processing or transport have been generated.

The participating groups analyzed the function of key proteins with different subcellular localization that were assumed to play a role in lipid entry pathways. Among them are proteins with cytoplasmic localization or orientation, especially the oxysterol binding protein-related proteins (ORPs) and rab-proteins. Among the proteins with endosomal and lysosomal localization were the Niemann-Pick disease, type C-proteins, and the saposins. Cells from human patients were analyzed, and also phospholipidoses as induced by drugs, or lipotoxic lipids were a major focus of this initiative.

Lipid Probes

Lipid probes labeled with chemical reporter groups, isotopes, or fluorescence were prepared and/or analyzed by the participating groups. For example, a fluorescent cholesterol derivative was used as novel tool to determine kinetics of cholesterol uptake and intracellular transport. It was demonstrated that sterols are transferred from the plasma membrane to lipid droplets via the Endoplasmic Reticulum, and not directly (Jansen et al., 2011). As another fluorescent lipid probe, a ganglioside bearing a fluorophore in a position that does not alter membrane anchoring was prepared. This lipid probe was suitable for STED-microscopy (STED = Stimulated Emission Depletion) and enabled the characterization of so-called lipid rafts as transient structures, which are discussed as starting points for endocytic uptake of certain lipids. With the aid of this probe, an upper limit of lifetime and size of such domains in the range of 20ms and 20 nm was found (Eggeling et al., 2009). Several additional lipid probes have been prepared and were e.g. used to analyze the function of the 4,5-E-double bond present in most sphingolipids (Brodesser & Kolter, 2011).

Roles of proteins and membrane lipids in cellular lipid uptake pathways

ORP-Proteins

OSBP-related proteins (ORPs; ORP1-ORP11 with several sub-forms) are binding proteins for oxysterols (Olkkonen & Hynynen, 2009; Olkkonen, 2012; Olkkonen et al., 2012). The function of this group of proteins is largely unknown; several human ORPs and their impact on cellular lipid entry pathways and on lipid delivery to lipid droplets was investigated within WP6.

The tools developed by WP6 enabled insight in the function of different proteins on cellular lipid entry pathways at the molecular level. For example, ORP2 was identified as a lipid droplet-associated member of the ORP family with impact on cellular metabolism of both triglycerides and cholesterol esters (Hynynen et al., 2009). Later, it was shown that for the first time in human cells that ORP1S and ORP2 play a functional role in sterol entry to lipid droplets. Recent data indicate that ORP2 operates at membrane contact sites via interaction with the endoplasmic reticulum (ER) protein VAP-A and lipid droplets.

It was further demonstrated that ORP1L operates at membrane contact sites between the late endosomes / lysosomes and the ER, where it controls the efflux of lipoprotein-derived

cholesterol from macrophage foam cells (Vihervaara et al. 2011). Binding of a sterol ligand by ORP1L regulates its affinity for VAP-A, which holds true for most ORP family members (Weber-Boyvat et al., manuscript in preparation). Based on this, a model was derived that in lipid/sterol depleted cells ORPs act to reinforce membrane contact sites and thus facilitate lipid transport between the ER and other organelles to ensure appropriate organelle lipid composition and functionality. The relevance of this for lipid homeostasis in vivo was illustrated by the fact that the 1st viable ORP knock-out mouse model, that of ORP8 displays increased high density lipoprotein (HDL)-cholesterol and phospholipid levels (Béaslas et al., submitted).

The work generating functional evidence for ORP function at organelle interfaces and for the function of membrane contact sites in lipid transport, organelle motility/positioning, and cell signalling was a major contribution towards the goals of LipidomicNet and has far-reaching implications for future medical applications.

Also the function of ORP7 (Zhong et al., 2011), ORP8, which is abundant in macrophages (Béaslas et al. 2012) and adipocytes (Zhou et al. 2012), and ORP11 (Zhou et al., 2010) have been addressed. Hepatic overexpression of ORP8 reduces serum and hepatic lipid levels in mice transduced with ORP8 adenovirus (Zhou et al., 2011). ORP8 targets the ER with a trans-membrane segment and the plasma membrane (PM) with its pleckstrin homology domain region, and recent results suggest that the protein, especially its non-sterol bound conformation, localizes at the ER-PM contact sites implicated in lipid trafficking and calcium regulation (Vihervaara et al., manuscript in preparation). Prompted by these findings, the impact of ORP8 silencing on the macrophage lipidome (Vihervaara et al. 2012), transcriptome, and migration behaviour (Béaslas et al., 2012) was investigated. The results demonstrated modification of the cellular free cholesterol and cholesterol ester content in ORP8 knock-down macrophages, and a number of subtle alterations in the macrophage glycerophospho- and sphingolipid content, as well as enhanced migration capacity. To elucidate the in vivo function of this interesting, putative lipid uptake regulator, the 1st viable oxysterol binding protein knock-out mouse model, that of ORP8 (Béaslas et al. 2012, submitted) was generated within WP6.

Furthermore, ORP10 was identified as a regulator of hepatic cell lipogenesis that localizes on microtubules and may thereby affect organelle structure/positioning or membrane trafficking. Genetic studies demonstrated the role of the OSBPL10 gene in determining high serum triglyceride levels in Finnish dyslipidemic subjects (Pertilä et al., 2009). A functional role of ORP10 was found as a modifier of apoB-100 lipoprotein secretion by human hepatocytes (Nissilä et al., 2012).

Expression patterns and function of ORPs was also analyzed in adipocytes. ORPs are expressed at high levels in adipose depots, and induced (ORP11) or down-regulated (ORP2, 3, 4, 7, 8) during Simpson-Golabi-Behmel syndrome cell adipogenic differentiation (Zhou et al. 2012).

Endolysosomal proteins

The late endosomal membrane protein NPC1 plays a key role in endo-lysosomal lipid handling, and affects the storage of triglycerides in lipid droplets. In NPC1-deficient mice, hepatocyte triglyceride content and secretion is markedly reduced, and cholesterol synthesis is dramatically increased. Also common genetic variation in the NPC1 gene contributes to serum triglyceride levels in humans (Uronen et al., 2010). It was also investigated how the drug fingolimod (FTY720), which is used in the treatment of multiple sclerosis, affects

macrophage foam cell formation. Fingolimod increased NPC1 and ABCA1 protein levels in macrophages, and thereby increased the delivery of cholesterol from endo-lysosomes to apoA-I, while decreasing its delivery to lipid droplets (Blom et al., 2010). These findings suggest that FTY720 has potent cardiovascular effects and that it may exert atheroprotective functions.

In addition to the NPC1-protein, endolysosomal lipid transfer proteins were investigated within WP6. The four saposins A-D are membrane-active lysosomal lipid transfer proteins that are required for different steps of glycosphingolipid degradation and for the loading of lipids on CD1-proteins of the immune system. The NPC-2-protein transfers sterols on NPC-1. These glycoproteins were prepared in pure form and applied to newly developed lipid transfer and membrane fusion assays for the functional characterization of these proteins (Abdul-Hammed et al., 2010). Based on the results obtained with the aid of these assays, a model for membrane lipid sorting within the lysosomal compartment could be established (Sandhoff & Kolter, 2010; Gallala et al., 2010).

Diseases associated with lipid uptake pathways

Impaired lipid entry pathways are associated with human diseases and with the formation of lipid storage organelles, such as the lamellar bodies. This can be caused by genetic defects, metabolic overload or can be drug-induced. The impact of different proteins on lamellar body formation was investigated in the human skin (Frateschi et al., 2011; Fehrenschild et al., 2012). Lipid storage induced by modified lipoproteins was demonstrated as a new mechanistic principle for phospholipidosis (Schmitz & Grandl, 2009). In macrophages, endolysosomal and cytoplasmic lipid storage is induced by oxidized LDL (Ox-LDL) and enzymatically degraded LDL (E-LDL), respectively. Ox-LDL is resistant to rapid endolysosomal hydrolysis and is trapped within the endolysosomal compartment. This leads to the generation of lamellar bodies, which resemble the characteristics of phospholipidosis. In contrast, E-LDL leads to cytosolic lipid droplet accumulation and moderate upregulation of ABCA1 and ABCG1 (Schmitz & Grandl, 2009; Grandl & Schmitz, 2010). This has an impact on macrophage foam cells in the arterial intima during atherogenesis. Transcriptomic and lipidomic analyses in cultured cells that were challenged either by endolysosomal phospholipidosis induction or by lipid droplet induction were carried out (Orsó et al., 2011). Based on studies in cells and plasma, BMP and oxysterols were demonstrated as lipid biomarkers for Niemann Pick disease, type C1 (Orsó et al., manuscript in preparation).

Bis(monoacylglycero)phosphate, a key lipid for endolysosomal membrane degradation

A key factor for endolysosomal membrane degradation is the unusual lipid bis(monoacylglycero)phosphate, BMP (Schulze et al., 2009; Kolter & Sandhoff, 2010; Gallala and Sandhoff, 2010). BMP plays a critical role for human diseases associated with defects in cellular lipid uptake via endosomes and lysosomes (Schulze & Sandhoff, 2011; Kolter, 2011). Based on the methodology developed within WP2 (e.g. Scherer & Schmitz, 2011), the phosphatidylglycerol (PG) – bis(monoacylglycero)phosphate (BMP) – cardiolipin (CL) axis could be identified as a critical determinant of membrane traffic and metabolic balance in macrophages and the metabolic relationship between these lipids (Orsó et al., 2011).

Lipid entry pathways are impaired in genetic human diseases of (glyco)sphingolipid metabolism (Schulze and Sandhoff, 2011; Kolter, 2011). The role of the heat shock protein Hsp70 in Niemann Pick disease, type A/B, was investigated, and it could be demonstrated that Hsp70 stabilizes lysosomes and reverts Niemann-Pick disease-associated lysosomal

pathology via interaction with BMP (Kirkegaard et al., 2009). Also the role of arylsulfatase A and saposin B in another lysosomal lipid storage disease, Metachromatic Leukodystrophy, was further analyzed. The study revealed e.g. the requirement of unexpected high levels of saposin-B for maximum sulfatide turnover (Matzner et al., 2009).

Drug-induced phospholipidosis

To characterize lipid uptake pathways in cultured cells, uptake and metabolism of different isotope-labeled lipid probes was investigated in four different types of cultured cells, human skin fibroblasts, RAW264.7 macrophages, HepG2, and A431 cells. The influence of representative cationic amphiphilic drugs (CADs) on uptake and processing of the phospholipid probes was studied. For example, in macrophages, all investigated drugs lead to an impaired processing of exogenously added sphingomyelin- and phosphatidylcholine-probes. Also remarkable and cell type-specific effects of the antidepressant CAD desipramine on the processing of fatty acids were found (Hameed et al, manuscript in preparation).

Enabling Technologies: Mass spectrometry

Several genetic diseases arise from defects in glycosphingolipid and sphingolipid catabolism (Schulze & Sandhoff, 2011; Kolter, 2011) and lead to impaired lipid entry via this compartment. Lipidomics of glycosphingolipids (Farwanah & Kolter, 2012) is much less advanced than the determination of other lipid classes. Therefore, WP6 addressed also this topic.

A recently developed method allows the determination of the neutral glycosphingolipids glucosylceramide, lactosylceramide, globotriaosylceramide, and globotetraosylceramide by a combination of HPLC, densitometry, and atmospheric pressure chemical ionization (APCI) mass spectrometry. Also cholesterol and sphingomyelin are determined after one single HPLC separation together with the neutral glycosphingolipids (Farwanah et al., 2009; 2011).

In general, the quantitative determination of analytes by mass spectrometry requires suitable calibrating substances, either as external standards, or chemically modified as internal standards. Due to their structural complexity and to the lack of suitable standard substances, glycosphingolipids are not easily determined by mass spectrometric methods (Farwanah & Kolter, 2012) used within Lipidomics (Kolter, 2012). Glycosphingolipids are sialic acid-containing glycosphingolipids (Kolter, 2012) and implicated in a variety of genetic and acquired human diseases (Schulze & Sandhoff, 2011; Kolter, 2011). They are especially abundant in the human nervous system, and to a lesser extent in plasma and in many organs. Within WP6, internal standard substances have been prepared for the major brain gangliosides, GM1, GM2, GD1a, GD1b, GT1b, and GQ1b. The calibrators contain fatty acids with chain lengths of C14 that are not present in the endogenous samples. In addition, standard substances for the tumor-associated gangliosides GM2 and GD2 have been prepared by β-galactosidase-treatment of the GM1- and GD1b-standard, respectively (Gantner M, et al.; manuscript in preparation).

Work progress and achievements during the period for WP7

• *A summary of progress towards objectives and details for each task;*

The tasks performed by the WP7 WP in LipidomicNet can be split in the following sub-tasks:

1)- Definition and implementation of the different standard processing procedures. During the first year of LipidomicNet, the different task forces were assembled (D7.1) and different standard processing procedures were defined (D7.2.1-D7.2.4), implemented (D7.3.1-D.7.3.4), and the corresponding pipelines for data processing were set-up (D7.4.1-D.7.4.4). The data types covered were lipidomics/metabolomics (D7.2.1, D7.3.1 & D7.4.1), microarray and RT-PCR (D7.2.2, D7.3.2 & D7.4.2), protein mass spectrometry and protein chip (D7.2.3, D7.3.3 & D7.4.3), and fluoresce microscopy data (D7.2.4, D7.3.4 & D7.4.4). All the standard processing procedures were made available by month 12 in the LipidomicNet wiki (D7.5 http://www.lipidomicnet.org.) These procedures, and processing pipelines have been updated and interlinked in the wiki throughout the project.

2)- Curation of lipid specific pathways (D7.6). This work was done by different partners. First of all, Gottingen University (P17) was responsible of the development of the EndoNet database. This is an information resource that keeps information about intercellular communication events (http://endonet.bioinf.med.uni-goettingen.de). The evolution of data contents in EndoNet is summarized in Table WP7.1. At the start of the project, the low number of links between hormone and receptor (intercellular links) and between receptor-cell-combination and secretion of the next hormone (intracellular links) made impossible to perform a network analysis of the endocrine network. To summarize, a total of 14 pathways were available by December 2012. A new version of the EndoNet web interface will be available shortly after the end of LipidomicNet.

	March 2008	**March 2011**	**October 2012**
Hormones	967	1017	1014
Receptors	711	782	767
Tissue/Cells	401	405	406
Intracellular links (Influence)	238	489	518
Intercellular links (hormone-receptor)	866	977	1040
Phenotypes		474	529
Links to Phenotype		724	862

Table WP7.1. Data contents in EndoNet, from March 2008 till October 2012.

In parallel, EMBL-EBI (P16) performed curation of lipid-related pathways for Reactome (http://www.reactome.org). The following relevant pathways were added:

a) Glycerophospholipid Biosynthesis pathway. It is available here:

http://www.reactome.org/entitylevelview/PathwayBrowser.html#DB=gk_current&FOCUS_SPECIES_ID=48887&FOCUS_PATHWAY_ID=1483206&ID=1483206.

b) PI Metabolism pathway, including synthesis and transport of PIPs. It is available at:

http://www.reactome.org/entitylevelview/PathwayBrowser.html#DB=gk_current&FOCUS_SPECIES_ID=48887&FOCUS_PATHWAY_ID=1483255&ID=1483255.

c) Inositol Phosphate Metabolism pathway. It is available at:

http://reactomecurator.oicr.on.ca/entitylevelview/PathwayBrowser.html#DB=gk_central&FOCUS_SPECIES_ID=48887&FOCUS_PATHWAY_ID=1483249&ID=1483249.

d) Arachidonic Acid Metabolism pathway. It is available at:

http://reactomecurator.oicr.on.ca/entitylevelview/PathwayBrowser.html#DB=gk_central&FOCUS_SPECIES_ID=48887&FOCUS_PATHWAY_ID=2142753&ID=2142753.

e) Ubiquinol biosynthesis. It is available at:

http://reactomecurator.oicr.on.ca/entitylevelview/PathwayBrowser.html#DB=gk_central&FOCUS_SPECIES_ID=48887&FOCUS_PATHWAY_ID=2142789&ID=2142789.

f) Sphingolipid metabolism, including sphingolipid *de novo* biosynthesis and glycosphingolipid metabolism. It is available at:

http://www.reactome.org/entitylevelview/PathwayBrowser.html#DB=gk_current&FOCUS_SPECIES_ID=48887&FOCUS_PATHWAY_ID=428157&ID=428157.

g) alpha-linolenic (omega3) and linoleic (omega6) acid metabolism. It is available at:

http://www.reactome.org/entitylevelview/PathwayBrowser.html#DB=gk_current&FOCUS_SPECIES_ID=48887&FOCUS_PATHWAY_ID=2046104&ID=2046104.

BioBase (P18) also contributed to the pathway curation tasks. Overall, more than 300 metabolic reactions and 69 metabolic chains related to lipids have been created resulting in 22 new pathways. Additionally, lipid-related signaling data was also curated. One example for lipid-related signaling is the regulation of 'autophagy'. Currently, the autophagy pathway comprises 11 different signal cascades/chains. All the information curated within this project has been published as part of the regular releases of Proteome^{TM} database.

3) Public availability of the source data in standardized formats in public repositories (where available) (D7.7). P16 (EMBL-EBI) provided a document (called 'Recommended data exchange formats, ontologies and minimum reporting guidelines') that was sent to all the members of the consortium pointing out the main repositories and data standards available for the main types of high-throughput data generated within LipidomicNet: transcriptomics (microarray data), lipidomics (MS-based) and proteomics (MS-based). A training session was also provided at the LipidomicNet meeting in Vienna (July 2012). By December 2012, two datasets had been submitted to such resources by partners P06 (ETH) and P07 (Graz) (see details in deliverable report D7.7). However, at the moment of writing, different partners are still working on getting their submissions done.

4) Finalized LipidNet system with public Wiki access and documentation for users as well as collaborators (other databases such as Reactome, KEGG, and LIPID MAPS) (D7.8). The LipidomicNet wiki (www.lipidomicnet.org) was set-up and maintained by P01 (University Hospital Regensburg). By December 2012, the LipidomicNet wiki (with around 1,400 pages) had approximately 500 registered users. Presently there have been over 2 million page views. The restriction of belonging to a work-package, group or taskforce has been removed in order to have full access to the wiki. However, for sake of tidiness visitors are still required to register in order to view certain content. In addition to the LipidomicNet wiki, a framework for the storage of MS-based lipidomics data has been developed. This framework has three main components:

A)- A **standard nomenclature for lipid species**: A detailed proposal for a standardized nomenclature was done by some of the LipidomicNet partners, based on commonly, officially accepted terms, building upon the LIPID MAPS terminology (http://www.lipidmaps.org). Two levels of nomenclature are covered, for both low and high level resolution instruments. The proposal contains the following lipid families: Fatty acids (FA), Glycerolipids (GL), Glycerophospholipids (GP), Sphingolipids (SL) and Steroids (ST). As a result, a manuscript was submitted to *Journal of Lipid Research* (JLR) on December 2011. After two rounds of review, we decided to involve the International Lipid Classification and Nomenclature Committee (ILCNC, chaired by Prof. E. Dennis, also the head of LIPID MAPS). As a result, the proposal was further refined and the related manuscript was resubmitted to JLR on October 2012 and was finally accepted for publication on December 2012 (Liebisch *et al.*, *J Lipid Res*, in press).

- B) Creation of a **database of theoretically existing lipid species** ("LipidHome"). The aim was to build a database of the theoretically existing lipid species, which would be hopefully able to fill the existing gaps in LIPID MAPS, and more importantly, would serve as a common reference system to lipidomic researchers. Designed specifically to accommodate high throughput MS-based approaches, lipids are organized into the hierarchy defined by the LipidomicNet nomenclature, which reflects the variety in the structural resolution of lipid identifications. In the first release of LipidHome, theoretical lipids are generated for the "*Categories*" glycerolipids and glycerophospholipids.

In parallel to the database, a dynamic web application was developed to present the information and provide flexible computational access *via* a web service (http://www.ebi.ac.uk/apweiler-srv/lipidhome). The web application encompasses a browser for viewing lipid records and also a 'tools' section where an MS1 search engine is currently implemented. Additionally, cross-references to other lipid related resources (LIPID MAPS, ChEBI) were integrated and text-mining approaches were used to annotate lipids with relevant papers that mention them. By December 2012, a manuscript describing LipidHome has just been submitted to JLR (Foster *et al.*).

- C) **Standard data format for MS-based lipidomics results**. In order to report and store experimental results coming from MS lipidomics approaches, P16 (EMBL-EBI) led the development of the mzTab data standard (http://code.google.com/p/mztab/). mzTab is intended as a tab-delimited file, providing a comprehensive summary of MS-based identification and basic quantification results for proteomics and metabolomics. Thus, mzTab files can contain protein, peptide, and small molecule identifications together with experimental metadata and basic quantitative information. It is not intended to store the complete experimental evidence trail but only the final results. We additionally developed a free, open-source Java Application Programming Interface (API) to read and write mzTab files called *jmzTab*.

In order to formalize it, the mzTab data standard was submitted to the Proteomics Standards Initiative (PSI, http://www.psidev.info/) document process on June 2012. In parallel, a manuscript describing mzTab was submitted in July 2012 (Griss *et al.*) to *Molecular and Cellular Proteomics*. By December 2012 we are making the minor changes that are needed to comply with the reviewers' comments. We are planning to resubmit the standard and the manuscript by the beginning of 2013. Afterwards mzTab will formalized and the first stable specification will be fixed. An exporter from the *LipidDataAnalyzer* tool (developed by P07 in the context of WP2, PMID: 21169379) to mzTab was also implemented (from version 1.6.0, May 2012).

5) Data integration. P21 **(**Integromics) has been working towards facilitating this objective in their software *LipiMiner* for RT-PCR data. Two key functionalities are:

- A Universal data loader: A new custom data loader was created to be able to generically load data from all possible sources and qPCR machines manufactures.

- Pathways visualization and integration: The gene expression results generated from RealTime-qPCR data can now be visualized in a pathway context in compliance with the SBML/SBGN community standards (also used by the Reactome database).

In addition, P25 (Institute of Systems Biology) has carried-out the development of the open-source BioUML platform (http://www.biouml.org), towards the integration of metabolic quantitative data with gene expression and genome data. At the start of the project BioUML capabilities spanned the range including access to databases with experimental data, tools for formalized description, visual modeling and analyses of complex biological systems. During the project, the BioUML platform has been further developed towards tight integration of different types of “omic” data, new methods for their analysis and respective processing workflows, as well as providing possibility of joint remote work with data and, in such way, of performing collaborative reproducible research in consortium. In addition, to organize access and work of researchers in BioUML, the ‘BioStore’ system was also developed. ‘BioStore’ is aimed to be the single place where researchers can register and obtain access to various products and services (either free or commercial).

6) Data analysis. Some of the partners of WP7 have also performed the analysis of experimental data generated by other members of the consortium in the context of other work packages, thus facilitating the integration of experimental work and the bioinformatics analysis of the data. This work is summarized in the corresponding work packages.

Work progress and achievements during the period for WP8

The objectives of WP8 were related to the training of individuals involved in LipidomicNet, to the communication within LipidomicNet and beyond and to the dissemination of the achievements of the consortium for the benefit of the project. These objectives were tackled with support of WP1. Within the objectives of the project also were the opening and curation of the LipidomicNet public website and intranet, to launch press and update releases along with organization of the multiple LipidomicNet meetings and workshops and pursue a common publication policy agreed with the SC and executed by the MT. Very important was organization of consortium workshops and meetings every ½ year, in cooperation with the MT, the task forces, the SC and WP1.

WP8 has been engaged in the integration of scientific community transfer of information to society; demonstrating how stakeholders can exploit information if it is presented at an appropriate stage, using concepts/ approaches targeted to their needs, based on common methods, planning, and standardization of standard operating procedures. Thus, WP8 has maintained and advanced partner by supporting WP leaders' dissemination activities within the network.

Publications

Consortium as a whole published within the time frame of the project **213 scientific articles**. 448 posters or oral presentations were presented results of the consortium's achievement at scientific congresses, conferences, and workshops. Moreover, 9 papers are submitted and some are in submission process, and some more of publications are prepared for submission.

Within **WP1** being management work-package no manuscripts were published, but WP1 succeeded in dissemination of project related information at national and international level, focusing especially on EC partnering and disseminating events.

Partners working within **WP2** published 23 publications. WP2 LipidomicNet aimed to harmonize all existing preanalytical procedures and enabling high-throughput technologies. The main topics of publish publications are: lipid molecular species of the lipidome; transcriptomics/genomics of the lipidome; proteomics of the lipidome; high content, high throughput imaging; techniques for cell harvesting, isolation of lipid droplets and other cell organelles and also lipid droplet morphology.

WP3 was dedicated to perform study on mouse models. WP3 members published 22 publications concerning mainly lipid/protein interaction in liver and adipose tissue using different animal models; also in relation to lipid storage disorders; and lipid molecular species involved in lipid droplet biogenesis maintenance and degradation analyze retinoic acid signalling and depth study of targets involved in the regulation of lipid droplet formation.

WP4 aimed to study dysregulation of liver and adipose lipid droplet processing in human diseases published 60 publications. The main topics of this publications was lipidomic, transcriptomic and proteomic analysis of different tissues lipid droplets in humans, changes in gene expression in hepatocytes from T2D patients; gene expression changes in T2D or insulin resistant adipocytes; changes in signalling enzymes; identification of gene clusters related to influx/efflux and storage in energy overload diseases; changes in gene expression in HCV hepatocytes; lipid genes and protein analysis of LD during adipogenic progenitor differentiation; membrane microdomain imaging and analysis of lipid droplet; stress

associated changes in protein signalling and lipid droplet formation; lipid composition of lipid microdomains involved in lipid droplet assembly.

WP5 was established to study lipid droplets and lipid bodies in human macrophages and leukocytes. Partners working within this WP publish 20 scientific articles concerning: genes, proteins and lipid molecular species involved in lipid loading and deloading of macrophages; role of PAT proteins in the development of atherosclerosis; formation of macrophage derived foam cells; influence of normoxic and hypoxic conditions on lipid droplet processing; the role of the VLDL receptor in the hypoxia induced accumulation of lipid droplets in macrophages; interconnection between tissue macrophages and circulating monocytes and also influence of inflammation on leukocyte lipid body composition.

WP6 work concern mainly on cellular lipid entry pathways into lipid droplets and lamellar bodies. Partners published 74 articles concerning this topic. In papers authors delivered new knowledge concerning mainly: analysis of lipid entry into the different cells; lipid transport, processing, and delivery to droplets by receptor-mediated endocytosis or by direct transport; upgrade to high-throughput investigations with mass spectrometry as the read-out; regulation and modulation of lipid entry; role of different cell types (hepatocytes, macrophages, adipocytes) and proteins (oxysterol binding protein homologues, Rabs as well as lipid transfer proteins); regulation and modulation of lipid entry role of cationic amphiphils and other mediators of lipotoxicity; regulation and modulation of lipid entry pathway for drug-induced steatosis and phospholipidosis; lipid entry in cultured cells from human patients (obesity, metabolic syndrome, inherited diseases like Gaucher, Niemann-Pick, types A and C, Wolman, Farber); dentification of lipotoxic lipids in drug induced droplet formation; regulatory pathways relevant for generation of lipotoxic lipids; lipid delivery to droplets and development of cells expressing lipid binding proteins. Some of publications are prepared for submission.

WP7 was created to bioinformatic work to help other partners in interpretation of results from high-throughput instruments. WP7 partners published itself 20 scientific articles concerns definition of standard processing procedures for lipidomics/metabolomics data like: microarray data, RT-PCR data, for protein mass spectrometry and protein chip data; fluorescence microscopy data; implementation and setting up pipelines for processing of lipidomics/metabolomics data; creation of curated lipid-specific pathways, and also concern public Wiki access into Lipidomic database.

WP8 partners transferred information to society and scientific community by means of 12 press releases, AlphaGalileo subscription - access to the news service was provided from 2009 until December 2012; multiple oral and poster presentations at internal and external meetings and congresses and also by means of organization of LipidomicNet Master-class in Brussels in 2009 along with other internal events.

Unfortunately, it was not possible to include the following articles in the publications section of EC participants' portal (there only 210 publications are included):

1. Dönitz, J. and Wingender, E.: The ontology-based answers (OBA) service: A connector for embedded usage of ontologies in applications. Front. Gene. 3, 197 (2012).
doi: 10.3389/fgene.2012.00197
2. Farwanah, H.; Kolter, T., Lipidomics of Glycosphingolipids. Metabolites, (2012) 2, 134-164.
doi:10.3390/metabo2010134
3. Kolter, T.; Ganglioside Biochemistry. ISRN Biochemistry, (2012) 506160
doi:10.5402/2012/506160.

The 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 type 2 diabetes (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 $95 billion for health care.

EU-FP7 LipidomicNet (www.lipidomicnet.org) 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 SMEs) jointed their forces investigating lipid storage and release. The technical advances, high-throughput technologies, provided the optimal methodological possibilities to map the entire spectrum of lipids in cells, tissues and whole organisms.

The recent developments in lipid mass spectrometry 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 (www.biobase.de), Institute of Systems Biology (ISB) (www.systemsbiology.ru), ZORA Biosciences (www.zora.fi), Integromics (www.integromics.com) and Protagen (www.protagen.de) 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 organized as a detailed special purpose Wiki base available at www.lipidomicnet.org. LipidomicNet was linked and collaborated with the NIH initiative LIPID MAPS (www.lipidmaps.org) and the Japanese pendant Lipidbank (www.lipidbank.jp) as well as other European initiatives such as ENOR **E**uropean **N**etwork for **O**xysterol **R**esearch (http://oxysterols.com/). It is closely connected with the Danubian Biobank consortium (SSA DanuBiobank, www.danubianbiobank.de) for clinical lipidomics, DEEP (http://deutsches-epigenom-programm.de, 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 USA and Asia. Funding LipidomicNet and thus promoting the field of Lipidomics was of benefit for areas such as health, nutrition and disease management.

WP1. During the project runtime, it became obvious that without the Management work-package 1, the project execution would be really handicapped as LipidomicNet is quite a large-scale project uniting 25 partners and 3 sub-contractors. Thus the burden of administrative duties did not rest on the shoulders of scientist allowing them to concentrate on research.

The consortium was managed by Management Team (MT) and the fully devoted project manager Megi Sharikadze. Management Team consisted of Christoph Thiele (WP2 leader), Christian Wolfrum (WP3 leader), Michael Wakelam (WP4 leader), Juan Antonio Vizcaino (WP7 leader), Gerd Schmitz (WP1, WP5 leader & Coordinator). MT was actively supported by webmaster of LipidomicNet Richard Noragon, who was regularly updating LipidomicNet website according to decisions made. This team provided the immediate support to RTD program and adequately responded to changes occurring throughout the project lifetime.

Primarily, within this WP there was an emphasis on efficient communication and internal management of knowledge, on transparent and quantitative metrics used to ensure objective assessment of the Consortium’s progress.

The Management Team members got together during LipidomicNet internal as well as external events when there was a possibility. Apart from individual phone calls, the members communicated also on weekly/biweekly basis within phone and teleconferences. MT made important decisions concerning management of the material budget and overall issues of governance (ethics, financial, research, etc.). At the Steering Committee (SC) meetings, the MT reported to the SC. When needed WP1 also communicated with the external committees such as Audit and Ethics Committee, Intellectual Property Use and Dissemination Committee (IPUDC) and the Scientific Advisory Committee (SAC). The external advisors were invited to Consortium SC meetings and were subsequently asked to provide written their statements on project’s achievements.

WP1 largely facilitated the interaction between the scientific WPs to optimize combined evaluation of data obtained as well as to enhance possibilities of fruitful communication between partners involved in the consortium.

WP1 also allowed for a one-directional stream-lined communication with external stakeholders and EC. This one voice communication reduced excessive email traffic from beneficiaries to EC and vice versa: WP1 served as a central hub for project related information exchange related to e.g., internal and external event planning and organization, dissemination of EC project management knowledge and administrative tips, “how to”-s and lessons learned optimizing and facilitating consortium’s administrative activities. This was of benefit for local administrative groups with less experience in European Project management specifics. Many of WP1 activities left their foot-prints on LipidomicNet project Wiki as the project manager for the last three periods of project run, M. Sharikadze actively participating in population of LipidomicNet Wiki’s management and announcement board sections. After the formal closure of the project, this process will still continue and as a follow up, the summarized and finalized brochure on EC project management will be available for interested individuals.

The coordinating partner P01 UHREG, especially the Project Coordinator and Manager, extensively supported WP1 and WP8: Notably, management and scientific representatives of Regensburg and MT several times participated in EC health events stressing on promotion of LipidomicNet, on future directions of research via new consortium-building and grant submission. As a result, several grant proposals were submitted in the last years of the

project. The Project Coordinator took active part in dissemination activities intensively participating in national and international scientific events, promoting the consortium not only in Europe but in Asia (e.g., Russia, China, and Japan), Africa (Egypt) and America (USA, Canada). The LipidomicNet webmaster and PM, created, maintained, and published on the LipidomicNet Wiki a list of related international scientific events, especially emphasizing ones focused of participation of young scientists.

WP2. It is a general observation that progress in scientific concepts stimulates the development of novel methodology, which mutually delivers new insight that opens new avenues of research. As an integral part of LipidomicNet, method development has enabled or stimulated major parts of research in the network. But of course, the impact of new methodology developed in the consortium is way beyond LipidomicNet. Many research groups all over the world have used our methods or have adapted them to their specific needs. This is an ongoing process which will continue to influence concepts and results for many years.

WP3. It cannot been overestimated that standardization of lipid analysis in high throughput format for production of reliable and reproducible data sets has been achieved to a great extent in the LipidomicNet project. The concomitant competence attained in the different WP3 laboratories executing studies on mouse models has sparked high-end investments for mass spec and imaging instruments on the one hand, and exclusive sponsoring of MS meetings and an installment of a reference laboratory for CARS spectroscopy on the other hand. In addition, a patent has been obtained for “Lipid Data Analyser”, a bioinformatics tool for evaluation of lipidomic MS data. The competence in lipidomic analysis by LC-MS/MS has been applied to isolated LD from hepatocytes and adipocytes at lipid species level and lipid molecular species levels. The data reveal that nutritional stress (HFD, starvation) and genetic stress due to defects of proteins involved in LD metabolism result in phenotypic LD lipidomes, particularly of the triacylglycerols, and have diagnostic value for the diseases affected by the various stresses. Pertinent cooperations were not only carried out within the project, but also internationally with groups in Canada and Israel. In addition, LipidomicNet scientists obtained invitations to present the analytical progress made on a global scale.

Proteomic and transcriptomic screens for identification of keynodes have been carried out in parallel, to assess mouse models for diabesity that are best suited for the clinical situation. Thus, one group in cooperation with one of our SME partners and a specialist for lipid/protein interaction and with MS specialists identified a new ligand for a transcription factor responsible for protection from obesity and type 2 diabetes. A patent for the combination of this ligand and its druggable target has been filed. Examples for further keynodes identified by WP3 with the help of various screens is caveolin-1, responsible for correct LD morphology in adipocytes; TIMP-1 in the extracellular matrix of adipocytes turned out to be a negative regulator of adipogenesis in mouse *and* human physiology. By the same token, adiponutrin has been identified as a mainly lipogenic enzyme on hepatocyte LD, again in both mouse *and* human metabolism. Interestingly, a specific SNP of adiponutrin leads to liver pathologies (NAFLD) in either organism, the mouse model now being also *the* model to combat the human disease.

The combination of lipidomic data with SNP data from GWAS to recognize defect genes by specific lipid markers is a further highlight of the LipidomicNet project in its pioneering efforts. The enzyme in question is ATP10D whose defect promotes easy development of obesity and insulin resistance on HFD feeding. The model produced so far allows describing the function of the protein and eventually will unravel the mechanisms leading to the diseased state.

WP4 brought together studies upon human cell lines and patient cohorts to study pathological disorders of liver and adipose tissue which relate to defects in lipid droplet turnover. Notably the WP integrated studies upon human and murine systems. This is a key impact of LipidomicNet as many studies to date have looked at the two experimental systems in isolation and thus have not benefited from the integration. As an important outcome WP4 generated a significant number of high impact publications (see publications list) including in the Nature family of journals and the New England Journal of Medicine. These publications will have a lasting effect upon scientific methodology and scientific and clinical knowledge.

The siRNA screen of genes regulating lipid droplet turnover required the generation of LD540, a novel lipid dye. The screen identified a number of important hits of which two have particular interest – AUP1 and FLJ21280. The further study of AUP1 utilized the integration of WPs 3 and 4, as its further analysis benefited from liver-specific adenoviral gene mouse knock-down. These studies demonstrated that AUP1 is a new protein with functions that include ubiquitination and a role in droplet clustering, whilst FLJ21820 is a potential lipase located to the LDs. These proteins have the potential to be future therapeutic targets.

Studies upon the signaling pathways involved in LD turnover pointed to a key role for the fatty acid G-protein coupled receptors, notably GPR120 and potentially GPR40. This work raises the possibility that antagonists of these receptors may have the potential to modulate lipid droplet formation and thus could be considered as future anti-obesity therapies. Beyond the receptor, inhibitor and siRNA studies suggest that intervention at the level of PI3kinase and PLD pathways may also be beneficial. Notably these pathways are targets in the regulation of insulin signaling and coordination is suggested pointing to a further outcome of LipidomicNet being an appreciation of unexpected effects of inhibition of particular signaling pathways. Additionally, the study of HCV-infected cells and patients has confirmed the importance of lipid droplets in the replication of the virus and suggested that inhibition of lipid droplet formation would be a successful approach to treat HCV-infected patients.

The study of the patient cohorts in the AIDA/ Acarbose Type 2 Diabetes Study, Ludwigshafen Risk and Cardiovascular health (LURIC) and EUROSPAN studies, as well as additional cohorts from weight loss, oral fat load and obese prediabetic and diabetic patient studies has provided key information upon associations between lipid species, disease and disease susceptibility. Gene association studies identified candidate genes including ATP10D, FADS1/2/3 and ELOVL2 and lipid species such as C16:0/18:0/24:1. These are of potential importance but require further characterization. Studies upon adipocyte differentiation highlighted FADS5 (SCD1) as a key rate limiting step this may also be a key future target.

Patient cohort studies also identified adipocyte-expressed genes associated with obesity phenotypes which are in parallel associated with the free-living ambulatory activity. Thus we have postulated that the metabolically advantageous properties of an expanded gluteofemoral fat depot could be due to release of 16:1 fatty acids. The proportion of 16:1 in the NEFA fraction was strongly correlated with global insulin sensitivity. These studies further point to key therapeutic targets.

Transcriptomic analyses of human subcutaneous (SCfat) and visceral (VISfat) fat identified 525 genes showing lower expression in SCfat than in VISfat and 108 genes showing higher expression in SCfat, both in females and males. Classification according Biological Processes (GO) revealed that genes with lower expression in SCfat mainly are inflammation associated while genes with higher expression in SCfat are mainly development associated.

These identifications provide the basis for understanding but point to the need for further study to build upon this impact.

WP5. The work performed in WP5 highlights the precise lipidomic, proteomic and transcriptomic characterization of human monocyte-derived macrophages, granulocytes and cardiomyocytes. Biochemical pathways have been elucidated which are extensively involved in the pathogenesis atherosclerosis, coronary heart disease, obesity and metabolic syndrome.

It has been shown that during monocyte to macrophage differentiation the major cellular lipid classes switched from cholesterol in monocytes (SREBP2-dominated transcriptional regulation) to monounsaturated fatty acid-enriched phosphatidylcholines in macrophages (SREBP1-dominated transcriptional regulation). Suppression of SREBP-1c activity and inhibition of fatty acid synthesis prevented monocyte differentiation and phagocytic macrophage function. These regulatory mechanisms and pathways are fundamental in physiologic macrophage function.

Dissection of oxidized LDL (oxLDL) and enzymatically modified LDL (eLDL)-induced cellular phenotypes indicated that these two atherogenic LDL-modifications were coupled with two fundamentally different cellular lipid storage responses in macrophages. Oxidized LDL preferentially up-regulated scavenger receptors, required for their internalization, induced lipid storage in the acidic compartment resembling drug-induced endolysosomal phospholipidosis, parallel with increased cellular content of the endolysosomal signature lipid bis(monoacylglycero)phosphate (BMP), pro-apoptotic signaling and appearance of ceramide-enriched surface membrane microdomains. By contrast, challenge of macrophages by eLDL led to expanded cholesterol- and sphingomyelin-enriched surface membrane microdomains, up-regulation of diverse pattern recognition receptors required for phagocytosis of eLDL, parallel with extensive lipid droplet formation, increased ER-stress and membrane contact site formation for interorganelle trafficking and signalling, and enhanced cellular content of the mitochondrial lipid cardiolipin (CL). These cellular processes are key initial events in atherosclerosis, coronary heart disease, obesity and metabolic syndrome.

Lipid stress of granulocytes by eLDL induces a rapid apoptosis parallel with an extensive shedding of granulocyte-derived extracellular vesicles (EVs), which activate macrophages and contribute to the sustained low grade inflammation in atherosclerosis.

The hypoxia/ischemia-induced accumulation of lipids in cardiomyocytes depends on expression of the VLDL receptor (VLDLR) through a process that involves endoplasmic reticulum stress and apoptosis. The impact of VLDLR in the pathogenesis of myocardial infarction has been demonstrated, since inactivation of VLDLR (in global knock-out mice or by specific antibody) reverses cellular processes that are induced during hypoxia and/or experimental ischemia.

In addition to the impact in the cellular pathogenesis of diverse disease processes of public health disorders, the novel data generated by WP5 are suitable for development of software modules for further in silico analyses as working templates.

WP6. The impacts of the work carried out within WP6 are currently mainly at the level of the scientific community. Here, WP6 contributed to the development of several new concepts in research. Opinion leadership can be documented by a series of review articles and editorials that have been published on topics central for LipidomicNet objectives.

Elina Ikonen and Vesa Olkkonen have actively participated in developing and discussing new concepts in lipid cell biology: Vihervaara et al. (2011), Olkkonen et al. (2012a,b), Olkkonen (2012), Salo et al. (2011), Blom & Ikonen (2012).

Different aspects of lipid entry pathways and their impact on human diseases have been addressed by Gerd Schmitz, Konrad Sandhoff, and Thomas Kolter, such as the role of bis(monoacylglycero)phosphate (Scherer & Schmitz, 2011; Gallala & Sandhoff, 2011), the context of lipoproteins, lamellar bodies, and hyperlipidemia (Orsó, Grandl & Schmitz, 2011; Orsó et al., 2009; Schmitz & Grandl, 2009a,b), and genetic and other human diseases of endolysosomal lipid processing (Schulze & Sandhoff, 2011; Kolter, 2011).

This refers also to enabling technologies with relation to lipid entry, such as lipidomics of lyso-phosphatidylcholine (Schmitz & Ruebsaamen; 2010), plasmalogens (Wallner & Schmitz, 2011), (glyco)sphingolipids (Farwanah, Kolter & Sandhoff, 2011, Farwanah & Kolter, 2012), and gangliosides (Kolter, 2012).

In addition to their contribution to basic research, some of the results have significant potential in seeding emerging technologies and assessing therapeutic potential in medicine. Just to mention two examples: a new foreground with a definite application potential is the invention by Vesa Olkkonen and coworkers on a putative use of ORP8 as a therapeutic target, the manipulation of which could be employed for HDL elevation and novel treatments for cardiovascular diseases. Or the discovery by Elina Ikonen's group that the novel immunomodulatory compound undergoing clinical trials, FTY720, has major impact on human macrophage foam cell formation, warrants careful follow-up of its cardiovascular effects in man.

Other results summarized in the WP6-reports could provide new analytical methodologies and means of manipulating organelle/cellular lipid homeostasis and lipid-dependent signaling processes, with far-reaching implications for future treatment strategies of cancers, dyslipidemias, neurologic, and cardiovascular diseases.

WP7. Bioinformatics

The impact of WP7 can be split in three different categories: software, standards and biological data and knowledge disseminated to the community (through the wiki and databases).

1) Software. Most of the software tools that have been generated by the consortium are distributed as open source software, which ensures that third parties can fully benefit from it. This is the case of the "LipidHome" database, the BioUML platform (and the associated developed analysis pipelines) or the *Lipid Data Analyzer* (produced in the context of WP2). The exception to this general rule is the *LipiMiner* software, which has been developed by the SME Integromics (P21) to handle the analysis of Real Time-PCR data. In any case, all the developed software provides the lipidomics community with new bioinformatics tools, which are highly valuable due to the limited number of alternatives that are currently available in this nascent field.

2) Standards. Before the start of LipidomicNet, there was no data standard aimed at the reporting of mass spectrometry (MS)-based lipidomics results. In addition, the existing *de facto* standard nomenclature in the field (provided by the LIPID MAPS consortium) was not optimized for this type of approaches. Different LipidomicNet partners have been working together to address these limitations. As a result, the new data standard mzTab (http://code.google.com/p/mztab/) can now be used to report MS lipidomics identification and

basic quantification results in a tab-delimited text format (as exemplified by the exporter provided by the *Lipid Data Analyzer*). In addition, the LipidomicNet nomenclature can be used together with mzTab. The development of both the file format and the nomenclature have taken a long time ensuring that points of view coming from a number of members of the community are taken into account.

The first stable versions of both specifications are almost final at the moment of writing, but both standards will need to be updated in time. As for any community standard, the adoption pace will depend on the scientists (both in academia and industry) that are interested in their adoption. At present, a broad adoption of mzTab (which can also be used to report MS-based proteomics results) seems quite likely since there are a number of groups already using or planning to use mzTab. For instance, in the metabolomics context, it is already being used by the MetaboLights database (http://www.ebi.ac.uk/metabolights/), the new resource for metabolomics experiments at the EBI. In parallel, it is being used in the context of the FP7 COSMOS project ("Coordination of Standards in MetabOlomicS"), where different members of the Metabolomics Standards Initiative (MSI) are also represented.

3) Biological data and knowledge disseminated to the community (through the wiki and databases). At the moment of writing the LipidomicNet wiki (http://www.lipidomicnet.org, with around 1,400 pages) had already been widely used (with approximately 500 registered users) providing over 2 million page views. Since all the data access restrictions have now been removed, all members of the lipidomics community can now freely access all the information, including among other things, the standard processing procedures developed by the LipidomicNet task forces. The data types covered are lipidomics/metabolomics, microarray and RT-PCR, protein mass spectrometry and protein chip, and fluorescence microscopy data. In addition, a vast amount of data generated by the University Hospital Regensburg (P01) coming from the curation of existing literature is also freely available through the Wiki.

As a key point, new lipid-related pathways have been incorporated to popular publicly available databases that can be accessed by the community. This is the case of Reactome or EndoNet, which have been largely enriched in this type of information. Analysis pipelines can now freely use the information included in both resources. Additionally, some pathway-related data has been curated by the SME BioBase (P18) and added to the regular releases of the Proteome™ database, available for the clients of the company.

Finally, the long-term public availability of some of the data generated by LipidomicNet through the EBI resources such (and others like Tranche) can maximize the value of the funds provided since it will ensure that data can potentially be freely re-used in the future by all researchers in the field.

To summarize, the bioinformatics related work carried out in the context of LipidomicNet benefits or will benefit:

- Computational researchers. The new standards, tools, pipelines and resources generated contribute to improve research in the areas of MS-based lipidomics.
- Biology and biomedical researchers: As wiki or database users, they have now access to a great amount of new publicly available data from the lipidomics field.

WP8: Training, communication & dissemination

General training aspects

One key element of the *LipidomicNet* consortium was the development of a program for continuous professional education for young scientists, members of partner's organizations. One of the goals of the *LipidomicNet* consortium was a relevant training activity for young researchers. To reach this goal, workshops, progress meetings, and annual scientific meetings with oral and poster presentations have been organized. Young graduates have been taught cross the barriers between the classical discipline of clinical medicine, the basic sciences (like cell biology and genetics) and bioinformatics. In our consortium, several European expert groups worked together giving a unique opportunity to train and exchange in the rapidly developing field of enabling technologies, functional genomics and pathogenesis of disease development to better understand energy overload disorders. This kind of regular technical training was a novel and also of great interest for post-doc students who intend to pursue a career in the field of lipidomics in biomedical research and pharmaceutical or diagnostical industry.

The Management Team coordinated all training activities. Each training session was announced to the *LipidomicNet c*onsortium and others (e.g. explicitly to other EU networks and the respective specific societies). In addition, external students and young scientists were specifically targeted to join the training activities. We also endeavor to have one to two workshops per year providing the opportunity to the *LipidomicNet* scientists to work and learn in a team environment. The SMEs was encouraged to support these educational meetings.

Additionally many of the *LipidomicNet c*onsortium participants gave lectures for pre- and post-doctoral (PhD and MD) graduate students at their respective universities and medical centers. Specific and relevant aspects of the *LipidomicNet* project were introduced.

WP8 has been engaged in the integration of scientific community transfer of information to society; demonstrating how stakeholders can exploit information if it is presented at an appropriate stage, using concepts/ approaches targeted to their needs, based on common methods, planning, and standardization of standard operating procedures. Thus, WP8 has maintained and advanced partner by supporting WP leaders' dissemination activities within the network.

As a result of LipidomicNet project totally 213 scientific articles were published and some more are at different phases of publication process (9 papers are submitted, and some of publications are prepared for submission). Furthermore, 448 posters and oral presentations were presented at the consortium's internal as well as on external scientific events such as various scientific conferences, congresses and workshops.

In close cooperation with Project Manager and coordinating partner P01, considerable work was done in event management activities of the consortium, totally 17 internal events were conducted in 10 countries hosted by 11 different partners. Thus project results were shared with local scientific communities. Event relevant information was provided via Wiki webpage www.lipidomicnet.org.

Networking of LipidomicNet with the two other international lipidomic research projects LIpidMaps (USA) and LipidBank (Japan) have led to common objectives and global cooperations.

Multiple members of LipidomicNet were invited as experts on high level international conferences all around the world. Importantly, LipidomicNet consortium was hosting the 50th ICBL conference (1-5 September, 2009), www.icbl2009.de, a leading conference related to "fundamental investigations in all aspects of lipid research and related applications". A total

number of 235 scientists from 29 countries (6 continents) attended the meeting with 27 invited lectures, 23 short-oral communications and 84 poster presentations.

Also, participation in external events was encouraged and widely practiced. Among multiple oral and poster presentations, one has to extra point out

- LipidomicNet master-class in Brussels given by the project coordinator (November, 2009),
- LipidomicNet special session in EuroFedLipids 2012 Congress in Krakow (September 2012). Thus a special LipidomicNet funded session was included in the program (Section 1: Diabesity and Lipidomics. There along with the experienced researchers, a young scientist from Regensburg also gave an oral presentation. LipidomicNet Project coordinator, members of management (PM) and dissemination WPs actively participated in EC Health partnering and disseminative events held in Brussels, especially on the third and fourth years of the project (e.g., 9-10 June, 2011, 9-10 February 2012, and 29-30.05.2012). They have spread the project information to the interested scientific community. As a result, several new partners were found, based on new partnerships several new grant proposals were prepared and submitted. Nowadays, grant-seeking collaborative process is still in progress.

Collaborations with other EU-funded programs

The *LipidomicNet* members also developed intensive collaborations with other EU-funded programs including **HUPO** (Human Proteome Organisation – Medical proteomics), MolPAGE (Molecular Phenotyping to Accelerate Genomic Epidemiology), **ProteomeBinders**, ***Cardiogenics*** (identification of genetic roots of coronary artery disease by combining stepwise genome wide association studies with transcriptomic and functional genomic investigation of relevant genetic variants), **NUGO** (The European Nutrigenomics Organisation, www.nugo.org), **LIPGEN** (diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis, www.lipgene.tcd.ie), and the **Danubian Biobank Consortium** (SSA DanuBiobank). All of these are highly supportive for the objectives of *LipidomicNet* and look forward to strong collaborations.

Within the framework of dissemination and cooperation policy, a very successful cooperation was initiated with another European consortium - “European Network for Oxysterol Research (ENOR) and a joint workshop was organized on 19–20 November 2010 in Munich, Germany). In this 2-days workshop, members of both consortia presented their works and further discussed possible ways of united research. Furthermore, the workshop presentations merged in a special issue of Chemistry and Physics of Lipids devoted to this event “Analysis and function of oxysterols and other regulatory and lipotoxic molecular lipid species” available via ScienceDirect web-service:

http://www.sciencedirect.com/science/journal/00093084/164/6

In conclusion, it has to be emphasized that Europe has so far played a pioneering and leading role in the biochemistry and analysis of lipids and most of the leading mass spectrometry providers are European companies. These mass spectrometry based nano-scale and high throughput technologies combined with molecular imaging and modern information technology will certainly revolutionize our understanding of the complex interaction networks in a functioning cell and how lipids together with genes and proteins determine cellular functions in health and disease.