PROJECT FINAL REPORT

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Final publishable summary

1. Executive summary

After 3,5 years of effort, the FLIP consortium was successful in achieving the main objectives of this ambitious project. Many research projects have been funded and completed, considerably increasing our knowledge in the field.

In terms of clinical work the three major projects we would like to emphasize here are: the establishment of the largest European NAFLD cohort, the HCC Registry and the consensual and standardized histological classification for diagnosis and staging of NAFLD which are now ripe for multiple scientific analyses. Follow-up substudies of these projects are already underway.

Basic research work provided new insight into the mechanisms of progression and determinants of disease severity.

This sustained collaborative work resulted in the identification of novel epidemiological and genetic determinants for NAFDL both in adults and adolescents. Furthermore, the FLIP consortium allowed promising discoveries of the mechanisms of initiation and progression of NAFLD by unraveling and identifying metabolic, inflammatory and fibrotic factors some of which could be relevant pharmacological targets. For this purpose, multiple specific culture systems, new animal models and other specific biological tools were developed. Our work also improved the diagnostic & prognostic validation of serum markers for NAFLD, and the development of new non-invasive, imaging diagnostic tools that can be used in any radiology center with basic magnetic resonance imaging technology.

The results of studies performed on dietary and lifestyle habits of NAFLD patients are ready to be shared with “The high level Group on Nutrition and Physical Activity” and “the Diet, Physical Activity and Health –Eu platform for action”. Lifestyle and diet changes are a key success factor but also hard to implement and sustain. Therefore a priority of the FLIP consortium was to overcome this bottleneck of clinical practice by devising information and education web-based tools for the patients and the physicians. Importantly, specific studies have described and validated methods to identify obstacles to change and to assess the motivational status of patients, a major aspect of successful implementation of non-pharmacological therapies.

We are very confident that through the FLIP consortium we were able to lay the foundations for the future, large-scale collaborative research on NAFLD in Europe. The best demonstration that the project was successful is the fact that all members of the consortium are eager to continue this collaboration by creating a European Collaborative Research Network on NAFLD that is sorely needed and was inexistent before FLIP. New European centers of clinical excellence in this field are planning to join in this endeavor.

2. Description of project context and objectives

Non-alcoholic fatty liver disease (NAFLD) has become one of the top concerns for the practising hepatogastroenterologist due to the obesity epidemic and its potential to progress to advanced liver disease which significantly impacts on overall and liver-related mortality.

The aim of the FLIP (Fatty Liver: Inhibition of Progression) project is to understand and prevent the progression of liver disease in NAFLD. FLIP is a consortium of basic scientists and practicing clinical hepatologists with an established track record and focus on research into the underlying mechanisms and management of patients with NAFLD. Therefore FLIP provides a unique opportunity to assemble the largest European cohort of patients with histologically diagnosed NAFLD with clinical and epidemiological data and with biobanks of DNA, frozen liver tissue and serum. These will be used in a

wide range of collaborative inter-disciplinary research projects aimed at addressing key unanswered questions related to the mechanisms and consequences of liver injury in NAFLD and the development of novel preventive and therapeutic strategies.

The main outcomes of FLIP will be new insights in the progression of liver disease in NAFLD in terms of initiating mechanisms and patients at risk, innovative diagnostic methods particularly adapted for large-scale screening and prognostic evaluation, improved implementation of lifestyle changes.

The main expected deliverables are:

1. A consensual histological classification of NAFLD/NASH;
2. A prospective pan-European cohort of patients with standardized inclusion criteria and histologically proven NAFLD/NASH;
3. Novel epidemiological determinants for NAFLD in adolescence and adulthood;
4. Clinical correlates and mechanisms of progression in NAFLD (spanning all the steps of the disease and answering key issues dealing with the interplay of metabolic, inflammatory and fibrotic factors in the initiation and perpetuation of liver injury);
5. Improved diagnostic & prognostic markers for NAFLD;
6. The implementation of preventive strategies in NAFLD.

In the long-term FLIP also aims to lay the foundations for the future of NAFLD research in Europe and create a European Collaborative Research Network on NAFLD for sustainable scientific progress in the next decades.

By disseminating the project's results, FLIP will further help the European Community to suggest guidelines on the management of this emerging liver disease.

3. Description of the main S&T results/foregrounds

Prospective pan-European cohort of patients with NAFLD

We have developed the largest multicentric European, transnational prospective cohort of 668 well-phenotyped NASH patients with standardized inclusion criteria.

The consortium defined a detailed protocol of inclusion in the FLIP cohort and created an electronic Case Report Form available to all participants. It also **designed a centralized data management team handling the data** and proceeding with the statistical analyses. In addition, the consortium selected standardized questionnaires assessing quality of life, signs and symptoms, physical exercise, type and quantity of food consumption and adapted them to the different participating countries and languages. We also **developed a FLIP standardized DNA, serum and plasma biobank** and standardized the collection procedures among partners. An important amendment to the protocol has been made allowing for the long-term follow-up of patients included in the FLIP cohort with the aim of assessing the cardiovascular, neoplastic and hepatological outcomes of these patients and in particular to the progression of liver disease.

Most of the clinical and biological features that have been found to be associated with advanced liver disease in NAFLD are of limited predictive value. A main reason for this is that they are based on monocentric studies including a rather small number of patients with limited biobank resources and only including highly selected patients with increased aminotransferase values. It was therefore deemed necessary to have a much larger database of patients with suspected NAFLD, included based on a wide variety of clinical presentation profiles, from different centers spanning several countries and with a systematic assessment of the anthropometric, metabolic, hepatic (histological) phenotype

together with serum and DNA samples. A high number of patients provides adequate power to study multiple interactions between determinants of disease occurrence and disease progression; it also allows discovery and validation programs of biomarkers of disease activity.

We designed a prospective cohort including patients at risk of NAFLD i.e. presenting with ultrasound defined steatosis, and/or increased liver function tests, and/or metabolic risk factors (overweight, visceral adiposity, type 2 diabetes, arterial hypertension, dyslipidemia) but no well identified chronic liver diseases including alcohol consumption of ≤50 g per day. All patients had to had a liver biopsy and ideally DNA collection and serum collection. Centers were allowed to include the highest number of patients prospectively included since January 2010. In parallel, a retrospectively collected cohort, following the same protocol was included, when available.

A total of 668 patients were included from 9 centers (Paris, Sevilla, Newcastle, Bologna, Torino, Modena, Sao Paolo, Bern, Wien) through a central e-CRF. Data quality was enforced by a central data manager. A central review of the slides was also organized in order to reduce interobserver variability and homogenize the reading using the new SAF-FLIP histological classification. The population was very homogenous and was clearly distinct from a population of alcohol drinkers: two thirds of patients never drank other than very occasionally; 9% were former drinkers and 24% only were current drinkers. Our data show that **there is an important association between age and the HOMA score (Homeostasis model assessment of insulin resistance, used to quantify insulin resistance) on one side and advanced fibrosis on the other side.** On the basis of this observation we developed an algorithm of risk stratification for an individual patient that can be used at the bed-side and complemented with non-invasive tests of disease severity.

This European cohort will also serve for numerous other clinical research subprojects of FLIP. Moreover, the inclusion in this multicentric cohort will continue beyond the funding period. In that sense we designed a prospective follow-up of this cohort based on yearly visits up to year 5. The aim of this follow-up study is to evaluate the potential for metabolic complications (occurrence of type 2 diabetes), cardiovascular complications (arterial hypertension, coronary heart disease, stroke, etc.), neoplastic complications and hepatic disease progression (changes in non-invasive markers/imaging (elastometry), fibrosis progression and/or NASH occurrence/disappearance on control biopsies at year 5, cirrhosis occurrence). This prospectively designed cohort, with mid-term follow-up will provide eagerly awaited data on the dynamics of the progression of NASH and its complications. Plans for large-scale collaborations with the NIH-funded NASH CRN are underway.

- **Consensual histological classification of NAFLD/NASH**

We developed a novel validated and consensual scoring system for liver biopsy interpretation that can be applied in the context of fatty liver disease for diagnosis system among the pathologists. The goal was to find a compromise between the development of a simple easily applied system (for making a firm diagnosis in individual patients, even when applied by non-specialists) and of a more reliable and discriminant system (for the application in large clinical trials).

A histopathology consortium of eight members, all European leaders in hepatic pathology was established which set-up several face-to-face meetings and web-conferences. The group started to develop a diagnostic algorithm for the diagnosis and staging of severe forms of NAFLD.

The new histological classification was finalized and then tested for reproducibility both within an expert group of liver pathologists and within a larger group of non-specialized pathologists. Our comparative study show that **the final algorithm allows general pathologists to reach a score of concordance as good as experienced liver pathologists, validating our approach and the new classification** (referred later as SAF score).

On the other hand, the classification was also tested in different animal models reproducing some aspects of NAFLD, but in these animal models the discriminative value of the SAF classification was

lesser than the one obtained in humans. This might be explained by the inability of the current animal models to reproduce the full morphological and metabolic spectrum of the human disease condition.

Investigations on the epidemiology of NAFLD

One of the key objectives of FLIP project is to identify reliable determinants of the occurrence of NAFLD in European population.

We therefore performed statistical analysis on distinct cohorts of patients allowing for different analysis:

- In Bristol, we have used data on over 2000 participants from the Avon Longitudinal study of Parents and Children (ALSPAC), a prospective birth cohort from the South West of England;
- In Copenhagen, we obtained permission to extract and use data from the national Danish Health Registries comprising all hospital admissions in Denmark from 1977-2011 (only data for all persons with a narrow range of diagnoses of liver diseases) and from several large Danish cohorts (Danish National Patient Registry and the Psychiatric Central Research Registry, but also Copenhagen School Health Record Register and the Helsinki Birth Cohorts). By linking the registers and the cohorts, we have made databases comprising 370.000 children and 165.000 adults with measures of body size, physical activity and NAFLD, and for the children also liver cancer;
- In Campogalliano and Cormons, follow-up examination of 40-70 years old male patients of selected high-, medium- and low-risk segments of the Italian Dionysos cohort have been conducted. In total, 68 participated out of the 123 patients which were invited.

Our main findings from the work done on ALSPAC cohort are that:

- Amongst otherwise healthy adolescents (with no diagnosed hepatic disease and with those who reported hazardous alcohol consumption removed), only 2.6% have ultrasound scan detected NAFLD, and this prevalence is similar in females and males;
- Birthweight and weight gain in early infancy (adjusted for length/height) are not associated with NAFLD, but from age 3 years onwards greater weight for height increase up to age 10 years is associated with greater total fat and NAFLD at age 17-18 years;
- Adolescents with ultrasound scan detected NAFLD are at increased risk of insulin resistance and liver fibrosis;
- High levels total energy intake at age 3 years and greater increases in energy intake up to age 7 years are associated with greater risk of NAFLD, but individual macronutrients (fat, carbohydrate, sugars and protein) are not associated with later NAFLD;
- Higher levels of time spent in moderate or vigorous physical activity at ages 11-12, 13-14 and 15-16 years are associated with greater risk of NAFLD at age 17-18 years with associations strengthening as the age of physical activity assessment increase;
- We have replicated previous findings in European adults of strong associations of specific genetic variants in this study of European adolescents. We also found evidence that genetic variants that have been shown to be robustly associated with ALT and GGT in genome wide association study (GWAS) of adults are also associated with these measures in adolescents. However we found no novel GWAS hits for shear velocity, ALT, AST or GGT at genome wide significance ($< 10^{-8}$).
- We did not find any strong evidence that either ALT or GGT are causally related to insulin resistance or associated cardiometabolic risk factors.

The main outcomes from the work done on Danish cohorts are:

- The results on the national time trend analysis suggest that in spite of the continued increase in prevalence of obesity in this country, the data do not suggest an increase of clinically recognized adult NAFLD in the Danish population. The results do not seem to be influenced by hepatitis, alcohol intake or the raising age-range of the population. However this would need further investigations as we suspect on the other hand that the change in the international classification of Disease from 8th revision to 10th revision (ICD10 coding system) may have an impact.
- The results on the 370.000 children suggest that:
    - Higher BMI (for Body Mass Index, a measure for human body shape based on individual mass and height) in childhood increases the risk of adult liver cancer (hepatocarcinoma or HCC can be considered as the worst step within NAFLD spectrum);
    - Higher BMI in childhood may increase the risk of adult clinically recognized NAFLD, but the association was not as clear as for liver cancer;
    - Weight gain during childhood may increase the risk of adult clinically recognized NAFLD independent of attained weight. Indeed, we observed that an important variation of BMI during childhood appear more important than the level of BMI in relation to the risk of clinically recognized NAFLD in adulthood.
- The results on the 165.000 adults suggests that:
    - Higher BMI and waist circumference increases the risk of each stage of clinically recognized NAFLD;
    - Higher HC may reduce the risk of each stage of clinically recognized NAFLD;
    - Weight gain during adult life increases the risk of clinically recognized NAFLD;
    - Physical inactivity may increase the risk of each stage of clinically recognized NAFLD, but the association was not as clear as for BMI.
- Our analysis of the 276,589 children from Copenhagen and Helsinki cohorts suggest that low birth weight may increase the risk of adult NAFLD.

Identification of genetic determinants of NAFLD

A cohort of ~2,000 DNA samples from biopsy of proven NAFLD/NASH patients has been collected from centers across Europe. Approximately half of the samples are from biopsies taken at the time of bariatric surgery the remainder are diagnostic biopsies taken in hepatology clinics. All patients have detailed phenotype data collected and harmonized across centers including co-morbidity (diabetes, insulin resistance, dyslipidaemia), blood biochemistry, anthropometrics and detailed histological analysis of diagnostic biopsy samples.

Genetic variation in a cohort of 1,125 European and North American Caucasian patients with histologically characterized NAFLD from the FLIP Consortium and the NASH CRN was determined. **For the first time in a histologically based GWAS we confirmed that single-nucleotide polymorphisms associated with the PNPLA3-SAMM50 locus are independently associated not only with steatosis but also inflammation, ballooning degeneration and fibrosis. We also identified a number of novel loci that associate with one or more histological features of NAFLD including steatosis, inflammation and fibrosis.**

This comprehensive study of genomic variation across the histological spectrum of NAFLD has further established the overwhelming significance of the chromosome 22 PNPLA3-SAMM50 loci to

all aspects of NAFLD and has identified a number of novel candidate modifier genes that, once validated, will offer new insights into disease pathogenesis.

Validation of improved diagnostic & prognostic markers for NAFLD

Diagnostic value of non-invasive biomarkers of liver injury in NASH and of their prognostic value in patients with hyperlipidemia, diabetes or morbid obesity : Fibrotest, SteatoTest and NASHTest

The FLIP project allowed us to validate the diagnostic value of non-invasive biomarkers (Fibrotest, SteatoTest and NASHTest) in a large, independent, ethnically and nationally diverse cohort. Moreover, we also studied the prognostic value of Fibrotest in patients with hyperlipidaemia and diabetes.

- A validation study of the diagnostic accuracy of three panels of biomarkers, Fibrotest, SteatoTest and NASHTest has been conducted in a cohort of morbidly obese patients. We also measured the same biomarkers after successful weight loss induced by bariatric surgery.
- We undertook several prognostic studies testing the prognostic value of the FibroMax panel of biomarkers to predict overall mortality, liver-related mortality and liver-related events in these cohorts of patients at risk for NAFLD: patients with type 2 diabetes, patients with hyperlipidemia, and patients with morbid obesity.
- In several studies we tested the prognostic value of well validated serum fibrosis markers for liver-related events in populations at high risk of NAFLD mainly patients with type 2 diabetes and hyperlipidemia. The serum markers were tested prospectively in the pan-European cohort of biopsied patients with NAFLD. In parallel, several monocentric exploratory investigations have been done to study new markers or combinations thereof for the different histological lesions of NAFLD.

Development of a new imaging method based on an MRI technology to allow a radiological, non-invasive prediction of steatohepatitis (NASHMRi) and of fibrosis (FibroMRi) in patients with NAFLD.

For the validation, we built an estimation cohort and worked on dedicated algorithms using neural network analysis. The developed computational optical analysis of magnetic resonance images (DEMILI®) showed a high potential as a steatohepatitis predictor and allowed us to define fibrosis stage in NAFLD patients. Moreover, the FibroMRi process shows a high potential as a fibrosis stage predictor. It is a reliable and safe method that uses protocols applied in clinical practice and allows exploring the whole liver. FibroMRI does not need to be complemented with other non-invasive serum-based diagnosis methods because it has been established that they do not improve the results of the analysis. NASHMRi process shows a high potential as a steatohepatitis predictor. It does not need either to be supplemented with other non-invasive diagnostic methods to accurately predict steatohepatitis.

Development of an NMR-based technique for visceral fat quantification and of an elastometry based technique for adipose tissue fibrosis

Because in insulin-resistant NAFLD patients, visceral fat is the main source of free fatty acids delivered to the liver, we attempted to develop an NMR-based technique for visceral fat quantification. **We have shown, for the first time that adipose tissue fibrosis could be physically measured and is linked to liver fibrosis.**

We have analyzed a large cohort of morbidly obese patients and proposed a histopathological algorithm and scoring system for the evaluation of liver lesions in this population. Interestingly, we

have observed that, among 700 morbidly obese subjects, 16% have no liver lesions. Studies of these particular patients showed that they have an increased fat mass percentage and a decreased of the amount of subcutaneous adipose tissue fibrosis. On the contrary patients with severe liver fibrosis have an increase in the adipose tissue fibrosis. These observations prompted us to create a new non-invasive tool, based on elastometry, to physically evaluate the fibrosis of the adipose tissue. We confirmed that subcutaneous adipose tissue is predictive of weight loss induced by bypass surgery, and that patients with higher level of adipose tissue fibrosis at the time of surgery were resistant to weight loss.

- **Identification of clinical correlates and mechanisms of progression of NAFLD.**

An important research axis of FLIP project was to investigate the mechanisms of progression of NAFLD from steatosis to inflammation (fibrosis), cirrhosis and end stage liver disease like hepatocellular carcinoma (HCC). Therefore we explored several biological aspects of NAFLD, namely metabolic factors, inflammatory factors, and fibrosis factors and hepatic carcinogenesis.

- **Metabolic factors and NAFLD**

Contribution of the omental adipose tissue to the pathogenesis and progression of NASH lesions

- Several laboratory studies have been initiated to understand the contribution of the omental adipose tissue to the pathogenesis and progression of NASH lesions. Transcriptomic analyses of different adipose tissue depots have identified several adipokines that might play an important role in liver dysfunction associated with obesity. We compared the size of adipocytes and the macrophage infiltration between three functionally linked adipose tissue depots: the liver, the omental fat and the mesenteric fat, were compared. To better characterize the function of the adipose tissue and its relation to liver damage, we also evaluated the secretion level of a panel cytokines and chemokines in sera of non-diabetic morbidly obese women.
- In a cohort of morbidly obese women with or without NASH we have collected systemic and portal blood, subcutaneous, omental and mesenteric adipose tissue and liver tissue during bariatric surgery. We have demonstrated that there is an increase in CD68+ cell in visceral adipose tissue of NASH patients. In line with these data, we measured by multiplex the levels of a panel of cytokines in systemic and portal blood and find a new marker that could be implicated in NASH, which is CCL 22. We studied in which extent visceral adipose tissue is involved in the release of this cytokine.
- In addition, lipidomic analysis showed a particular lipid signature in systemic and portal blood involving phosphatidyl-glycerol species. To evaluate the implication of adipose tissues in this signature, we measured the lipidomic profile in the adipose tissue secretion media of the same patient. Strikingly we found a profile different from blood, which strongly suggested the involvement of the gut microbiota in the blood signature compared to adipose tissue.

Role of hepatic triglyceride lipases in the progression to NASH

- Because ATGL (PNPLA2) and adiponutrin (PNPLA3) were identified as key major metabolic lipases determining lipid partitioning in the progression of NASH, we investigated the role of hepatic triglyceride lipases in the progression to NASH. We created animal models for addressing the role of adipose tissue triglyceride lipase (ATGL) in the regulation of endoplasmic reticulum stress in the liver and in the susceptibility to NASH, and for determining whether fatty acids (FA) flux (released by ATGL-mediated lipolysis) from adipose tissue to the liver could impact on the development of lipotoxic liver injury. For this

purpose we compared liver injury in wild type and ATGL knockout mice upon feeding with Methionine and Choline Deficient diet (MCD diet, a common model for studies on NASH). Interestingly, although ATGL knockout mice lack the initial enzyme of triglycerides hydrolysis, MCD feeding led to weight loss and presumable increased lipolysis in both wild type and knockout mice. In parallel to comparable weight loss, there was no change in serum parameters of liver injury between the genotypes. These results show that lack of ATGL in the adipose tissue and the liver does not prevent lipolysis and consequently does not impact on liver injury due to increased FA flux from adipose tissue to the liver upon MCD feeding.

- While the insights for PNPLA2 are still restricted to mice (awaiting future exploration in humans), PNPLA3 is already an established genetic factor in humans with novel mechanistic insights derived from FLIP project. ATGL (PNPLA2) determines steatosis, inflammation and ER stress along the course of NASH. Part of these ATGL effects can be explained by providing endogenous fatty acids (FA) as ligands for the nuclear receptor PPARalpha, for the first time linking lipid partitioning to lipid signaling. Data from this project also indicate that **PNPLA3 not only determines hepatic triglycerides stores, but also FA saturation indices and mitochondrial energy expenditure, possibly explaining the association of genetic variants with progression towards NASH.**
- Bile acids (BAs) and proteins involved in BA transport (BSEP) and signaling (FXR) were identified as key regulators of hepatic lipid metabolism and inflammation, however without impacting on metabolic lipases. Notably, therapeutic BAs such as UDCA facilitate lipid partitioning and thereby may counteract lipotoxicity by upregulation of the desaturate SCD-1.
- Using high-field MR-spectroscopy we were able to non-invasively distinguish NAFLD from NASH and advanced fibrosis based on their spectra reflecting FA/TG ratio and membrane composition in addition to mitochondrial function (NADPH, ATP). **Interestingly, PNPLA3 variants correlated with FA saturation index and ATP content**.

Insulin resistance as a causal factor in the pathogenesis of NAFLD

- Insulin resistance has been consistently associated with NAFLD/NASH. Although insulin resistance can be an intrinsic defect in NAFLD it's unclear whether and to what extent it is a causal factor in the pathogenesis of liver damage. Therefore, we explored the contribution of the different sites of IR to liver damage in patients with NAFLD in a wide range of liver damage. In order to exclude the independent contribution of the single components of the metabolic syndrome (namely obesity, type 2 diabetes and dyslipidaemia), we selected non-obese, non-diabetic, non-dyslipidemic NAFLD patients. 42 patients were enrolled to be compared with 24 historical controls and assessed hepatic, peripheral and adipose tissue insulin resistance and pre-hepatic insulin secretion by stable isotopes technique in the fasting state. We observed an increase of insulin resistance in the adipose tissue and in the liver in NAFLD patients vs. controls, and an impairment of glucose clearance (a measure of peripheral, mainly muscle, insulin sensitivity). The degree of impairment was proportional to the degree of liver damage expressed as fibrosis stage (no fibrosis *versus* mild fibrosis *versus* severe fibrosis). Our findings suggest that, **at least in the absence of obesity and type 2 diabetes, the liver is an "innocent bystander" that suffer from IR in other sites and tries to compensate for it.** The increase in insulin levels in these subjects was mainly due to increased insulin secretion and impaired glucose clearance.
- We compared these parameters to the amount of subcutaneous, visceral and hepatic fat measured by MRI. While visceral fat was anyway increased in NAFLD subjects, independent of liver damage, both subcutaneous and hepatic fat had a stepwise increase proportional to liver damage. **These findings suggest that abdominal obesity is always an important factor in the development of NAFLD and subcutaneous fat act as a co-factor when it**

increases significantly. The extent of fat in the different sites was always correlated with insulin resistance in the adipose tissue, thus suggesting that, although visceral depots are important for the direct delivery of FFA to the liver, the contribution of an increase in subcutaneous fat should not be under-evaluated in these subjects, nor the contribution of VLDL production by a fatty liver.

- In NAFLD patients, insulin action was severely impaired in the adipose tissue and in the muscle. The extent of adipose tissue and muscle IR was proportional to the degree of hepatic necroinflammation and fibrosis, thus highlighting the importance of primary metabolic impairment in these tissues in the pathogenesis of NAFLD. In the liver, despite increased hepatic insulin resistance during fasting, insulin efficiently suppressed endogenous glucose production during OGTT, thus allowing the maintenance of glucose homeostasis. **The current results suggest that in NAFLD the liver is initially the target of metabolic defects primarily arising in other sites, namely in the muscle and in the adipose tissue, and that these metabolic alterations can contribute to the development of liver damage.** IR can also be found in chronic liver disease of various etiologies and is due to a down-regulation of peripheral insulin receptors by chronic hyperinsulinemia, due to reduced hepatic degradation of insulin because of intra-hepatic shunting. An unsolved question is whether in NAFLD, insulin resistance is the cause or the consequence of liver disease. In order to solve this issue, we evaluated insulin secretion and hepatic insulin clearance in our NAFLD patients. The results show that in NAFLD patients the high insulin levels occurring both in fasting and postprandial states are related to reduced glucose clearance in the muscle (peripheral insulin resistance), with a compensatory increase of insulin secretion by the pancreas, as it occurs in "classic" IR states (such as diabetes and obesity). Both peripheral insulin resistance and pre-hepatic insulin secretion were increasing progressively by worsening of liver damage. Hepatic insulin clearance was similar to control subjects in the postprandial phase. **These results indicate that NAFLD patients have a compensatory hyperinsulinemia by the pancreas to overcome reduced glucose clearance in the muscle, and that liver damage is the consequence rather than the cause of IR.**
- Insulin resistant states are characterized by a "dysfunctional adipose tissue", which leads to lipid accumulation in multiple ectopic sites, including the liver and the visceral area. Ectopic fat can contribute to liver damage. **We found that fat accumulation in the visceral area is an independent risk factor for NAFLD and that all NAFLD patients have increased visceral fat.** However, subcutaneous and liver fat, when increased, provide an independent contribution to liver necroinflammation and fibrosis that should not be underestimated.

Relationship between metabolic handling of an oral fat load and the degree of liver damage in NAFLD

- Clinical experiments have been conducted to determine the relationship between metabolic handling of an oral fat load and the degree of liver damage in NAFLD. We studied lipid handling after an oral fat load in NAFLD and control patients. Adipose tissue lipolysis and postprandial lipid storage contribute substantially to the liver triglyceride pool in NAFLD but the relative importance of free fatty acids (FFA) flux versus composition is a matter of debate. The relative importance of free fatty acids FFAs flux (lipolysis) versus FFA composition in the pathogenesis of liver damage in NAFLD was explored. We found that NAFLD patients with severe fibrosis had higher plasma concentrations of saturated fatty acids (SFA) with a depletion of polyunsaturated fatty acids (PUFA), particularly of linoleic acid, emphasizing the protective role of PUFA compared with the negative effects mediated by high proportion of SFA. The ratio of medium PUFA/SFA was inversely correlated with severe liver damage and insulin resistance in the adipose tissue. **These findings suggest a key role of PUFA, and linoleic acid in particular, as protective factors against liver damage and adipose insulin**

resistance in NAFLD patients. FFA composition changed minimally during a lipid load test, but we observed a significant decrease in palmitoleic acid (protective) that was paralleled by an increase in myristic acid (toxic) concentration. De novo lipogenesis (DNL) in the liver reflects an adaptation of the body to handle high-carbohydrate loads by converting excess carbohydrate to fatty acids and triacylglycerol (TG), which contribute to increased fasting and post-prandial plasma TG concentrations. **DNL is significantly increased in patients with NAFLD, particularly in those with severe liver damage**.

- Oxidative stress mainly due to increased lipid peroxidation has been involved in the development of NAFLD and its progression to steatohepatitis (NASH). Fasting small dense LDL concentrations as well as oxidized LDL were higher in NAFLD patients during the fasting state, indicating a state of chronic oxidative stress. During the lipid load, small dense LDL concentrations did not change compared to controls, while oxidized LDL were higher in NAFLD 2 hours after the lipid load. A similar pattern was observed after the oral glucose load. **This observation suggests that hepatic oxidative stress is part of a more generalized condition of chronic, whole body oxidative stress and determines changes in circulating lipids predisposing to cardiovascular disease**.

- **Inflammation factors and NAFLD**

Role of microbiota induced by feeding a high fat diet on liver fibrogenesis

Because dietary habits affect gut microbiota composition, and endotoxin produced by Gram-negative bacteria stimulates hepatic fibrogenesis, we analyzed whether changes in the microbiota induced by feeding a high fat diet may interfere with liver fibrogenesis. Mice fed a control or high-fat diet (HFD) were subjected to bile duct ligation (BDL) to induce fibrogenesis. This resulted in increased collagen deposition and higher density of infection in mesenteric lymphnodes, as an expression of bacterial translocation. Pyrosequencing revealed an increase in percentage of Gram-negative vs Gram-positive bacteria, with a marked increase in Gram-negative Proteobacteria in HFD-BDL mice. Expression of the inflammasome components was increased in the liver of fibrotic mice, but significantly reduced in the gut. Finally, microbiota transplantation of the microbiota of HFD-fed mice induced higher liver damage in mice fed a control diet.

Role and mechanisms of action of MCP-1

Because of its role in the hepatic fibrotic response to chronic liver injury, we initiated an experimental project aimed at identifying monocyte chemoattractant protein-1 (MCP-1) cellular sources, and their relative contribution to liver injury in rodent dietary models of NASH. We set up experiments to analyze the relative roles of MCP-1 expression by resident vs. bone-marrow derived cells in a rodent model of NAFLD, to understand in greater detail the inflammatory pathways underlying this disorder. Compared to normal wild-type (WT) mice receiving WT bone marrow, WT mice receiving knock-out (KO, mutant lacking MCP-1) BM had a similar phenotype in terms of body weight increase, fasting blood glucose, and the response to a glucose tolerance test or an insulin tolerance test. As expected, KO mice receiving KO BM had a reduction in body weight, and improvement in glucose tolerance and in the response to insulin. However, chimeric mice receiving WT BM on the background of MCP-1 KO had a phenotype more similar to the 'full' KO mouse than chimeric WT mice receiving KO BM. When expression of inflammatory factors, such as Interleukin-1beta, or Interleukin-6 was measured in the liver, both hepatic resident cells and bone marrow-derived cells appeared to contribute to activation of inflammatory pathways. In contrast, adipose tissue inflammation was more markedly dependent on resident than infiltrating cells.

Expression of MCP-1 and other proinflammatory factors was also markedly decreased by a natural alkaloid (berberine) commonly used as a food supplement. This resulted in a marked reduction of inflammation in mice undergoing experimental steatohepatitis. We discovered that the anti-inflammatory action of berberine is associated with inhibition of inflammasome assembly and function, in different models of liver injury including NASH, and that its action is mediated by an interaction with a purinergic receptor (P2X7).

APE1/ref-1 as a novel modulator of inflammation and fibrogenesis in NASH

APE1/ref-1 is a mediator of TNF-alpha and free fatty acid oxidative stress damage and induces an inflammatory cascade involving NF-kappaB and Interleukin-8 (IL-8). We studied its role as a novel modulator of inflammation and fibrogenesis in NASH. Inhibition of APE1/Ref-1 redox activity by the specific inhibitor, E3330 was efficient in blocking both basal and TNFalpha-induced activation of IL-8 expression in hepatic cells. This action occurs at the transcriptional level through blockade of redox-mediated activation of NF-kB. In addition, E3330 treatment is able to reduce fatty acid-induced increase in IL-8 expression.

In this set of experiments, we also developed a highly-reproducible model of cellular steatosis, to be used to investigate interaction with fibrogenic cells is needed. While hepatocyte-derived factors may target fibrogenic cells, such as stellate cells, through paracrine mechanisms, comparative experiments indicate that hepatocytes and stellate cells must be cultured in contact to obtain a maximal profibrogenic action, and that exposure of fibrogenic cells solely to conditioned medium is not sufficient.

Role of indoleamine dioxygenase (IDO)

We analyzed the role of indoleamine dioxygenase (IDO), an enzyme which converts tryptophan to active metabolites, such as kynurenine, which modulates innate and adaptive immunity and inflammation. We have employed 1-methyl tryptophan (1-MT), a specific inhibitor of the IDO pathway, which interferes with the conversion of tryptophan into kynurenine, in mice treated with the MCD diet. 1-MT administration lowered serum ALT levels, and dramatically reduced the intrahepatic levels of genes associated with inflammation. 1-MT also inhibited the development of fibrosis, together with a significant reduction in the expression of angiogenic cytokines and in reduced vascular density. These data indicate that **IDO may be a novel modulator of inflammation, angiogenesis and fibrosis during steatohepatitis.**

- **Fibrosis factors and hepatic carcinogenesis**

Role of myofibroblast populations

Investigations of hepatic stellate cell-derived and portal myofibroblasts (PMFs) suggest that both myofibroblast populations accumulate with different kinetics in NAFLD and exert specialized functions. The localization of PMFs suggested pro-angiogenic functions, which we confirmed *in vitro* and *in vivo*.

Role of VDR

As increasing evidence indicated that the vitamin D-VDR axis may be central in liver diseases, including NASH, the study of vitamin D receptor (VDR) knockout mice indicates that the vitamin D-VDR axis may be central in the development of steatohepatitis and associated fibrosis. In addition, we could show that VDR is critical in maintaining the integrity of the bile duct epithelium, and that the loss of VDR alters apical junctional complexes via EGFR, in this epithelium.

Role of Kruppel-like Factor 6 (KLF6) in the pathogenesis of NASH progression and hepatocellular carcinoma (HCC)

In order to identify factors involved in the development of hepatocarcinoma (HCC), the consortium set-up a registry of patients diagnosed with primary liver cancer occurring as a complication of NAFLD.

We generated specific antibodies and developed RNA/protein-based assays for the detection and quantification of cell-specific expression of KLF6 isoforms in human NAFLD and a mouse model of NAFLD. The main outcomes of our studies are :

- **The development of an animal model of NAFLD reflecting natural development and progression of disease**, through stages of simple steatosis, NASH, fibrosis and hepatocellular cancer. Cancers developed in 95% of mice in association with dietary induced obesity and impaired glucose tolerance and NASH;
- The consolidation of data supporting a **key role for KLF6 upstream in regulating development and progression of NAFLD**. Data from *in vitro* studies (expression suppressed by insulin in presence of high glucose, increased expression and nuclear translocation induced by oxidative stress) and *in vivo* in mouse and human studies (suppressed in association with high fat diet and steatosis, but increases in the presence of NASH/disease progression). Genetic data support roles for KLF6 SNAP in NAFLD progression, as well as fasting blood glucose.
- **The identification of KLF6 downstream pathways, as a regulator not only of hepatic insulin resistance, but also of adipose tissue insulin resistance.** Key targets identified include glucokinase (GCK), as well as its key interacting negative regulator, GCKR, and the cell surface sulfatase SULF2.
- The discover that SULF2, a KLF6 regulated target, is expressed in tumour activated stromal cells in HCC. Moreover **SULF2 was identified as an independent predictor of poor survival.**

Creation of HCC Registry

The consortium designed a Registry including prospectively collected cases of HCC in patients with NASH. Briefly, all cases with documented HCC in patients without chronic liver disease due to alcohol consumption, viral hepatitis, hemochromatosis, drug induced, autoimmune or genetic liver disease were included. Cases were then adjudicated as probably or possibly related to NAFLD or to truly cryptogenic cases. We then analyzed the carcinologic features, the metabolic comorbididities, the severity of the underlying liver disease.

This sub-project was considered of high scientific priority by a number of members outside the consortium that agreed to participate in the registry: 8 centres in Italy, 4 in France, one in Holland, one in Brazil, and one in Germany. This allowed the inclusion of 352 cases. A striking finding was the **very low prevalence of cryptogenic HCC (9%) and a very high prevalence of HCC in patients with possible/probable NAFLD (91%)**. Separate genetic analyses have shown that patients carrying the I48M mutation of the adiponutrin gene (PNPLA3), which was found to be the major risk allele for NASH in GWAS studies performed by the FLIP consortium, are at higher risk for developing hepatocellular carcinoma.

■ Improved implementation of preventive strategies in NAFLD

Primary and secondary prevention of NAFLD is centered on behavioral aspects. The studies of the FLIP consortium in this area have been carried out along three different lines: 1) we studied the effects of acute exercise on gene expression associated with fatty liver and disease progression (including

liver cancer development) in experimental animals fed either a normal or a high fat diet and show that regular exercise diminish the risk for developing cancer; 2) we could show that aerobic and resistance exercise are simple measures that are able to reduce liver fat accumulation in NAFLD patients; 3) we studied the habitual lifestyle of NAFLD patients, their motivation to change lifestyle towards healthier diet and habitual physical activity and developed a manual toolkit and a web-based program to help patients in changing their lifestyle.

Influences of physical activity on the underlying disease leading to HCC

We investigated whether physical activity influences the underlying disease leading to HCC. To address this issue, we used 2 different animal models and studied the effect of acute exercise in the hepatic transcriptomic. We discovered that acute physical activity results in important changes in the expression of mRNAs in the liver. Particularly striking were changes in the expression of genes in the p53 pathway, involved in cell cycle and apoptosis. By performing long-term experiments with mice exercising moderately for 30 weeks, we could show for the first time that regular exercise diminishes the risk to develop HCC. When the mice were fed a high fat diet the effect of exercise was lost, but surprisingly the mice under this diet tended to develop less tumors. The reasons for this observation remain to be understood. Nevertheless, mechanistically, under normal diet our results suggest that physical activity increases the activity of the AMPkinase which negatively regulates mTOR complex 1 via phosphorylation of Raptor. **Therefore, physical activity regulates negatively one of the major hepatocarcinogenic pathway. These data provide for the first time scientific evidence given reasons to motivate patients at risk of HCC to exercise regularly.**

Studies of the effects of physical activity in NAFLD patients were carried out by comparing the effect of aerobic and resistance exercise. The aim of these clinical studies was to describe the dose-response relationship of exercise on liver fat and its mediators in patients with NAFLD. Liver fat was reduced by 40% and liver enzymes (ALT, AST, and GGT) decreased following the exercise program. Glucose level control also improved and we observed a decrease in HOMA2-IR, an index of reduced IR. There was also a decrease in whole body fat mass and an increase in fat free mass in the exercise group, independent of change in body weight. In summary, **our data indicate that aerobic exercise improves the metabolic milieu and reverses steatosis.** Inside the FLIP consortium, resistance training has been tested during carefully controlled programs. Liver fat was reduced by 13% in the exercise group and 27% of the exercisers were no longer classed as having NAFLD (i.e. liver fat <5%). Glucose control improved with resistance exercise with a 12% reduction in the area under the glucose curve during an oral glucose tolerance test and a decrease in fasting glucose from high-normal values to perfectly normal values. Resistance exercise also increased fat oxidation compared with controls. All these improvements occurred in the absence of change in body weight or diet.

A tool kit for patients' education and a web-based lifestyle intervention

Because dietary and lifestyle changes are the first line therapy in NAFLD, we developed a tool kit for patients' education and a web-based lifestyle intervention. The effectiveness of a behavioral modification in patients with NAFLD was tested and lifestyle patterns in regard to stages of change in NAFLD patients was assessed with the aim to analyze the motivation to change at both the action and maintenance periods.

In general, the macronutrient composition of the diet and the amount of physical activity are not very different between NAFLD cases and the normal, control population. They were tested by validated questionnaires in large groups inside the FLIP consortium, as a prerequisite to implement significant lifestyle changes. There is a suboptimal adherence to Mediterranean Diet, with an excess of refined carbohydrates and low fiber intake. Motivation to change lifestyle, tested in a large Italian cohort, is weak: the majority of patients does not consider exercise as an essential component of treatment and are scarce motivated to engage in habitual physical activity. This is not the case of dietary restriction:

the majority of patients are well aware that they have to reduce energy intake to reduce liver fat, although there is a difference between what they do and what they should do. **To help patients with lifestyle changes – and therapists to intervene -, we created a manual based on 5 modules for education and counseling**. The toolkit developed in Italian language contains the principles of correct nutrition, a summary of the diseases due to unhealthy lifestyles with specific reference to NAFLD, recommendations to monitor and change the nutritional habits towards healthier nutritional intake and recommended physical activity, the potential advantages of behavioral changes. The toolkit was created initially in Italian and later trans-cultural translations into Spanish and Danish were provided.

To increase the educational/behavioral support to NAFLD patients a web-based lifestyle intervention was created. The program (see http://www.sanistilidivita.eu) is divided into different modules (food pyramid; food labels; food intake reduction; physical activity), all preceded by questionnaires to test motivation and pre-study knowledge. Depending of the results, a dietician will provide feed-back on food diaries and patients will receive periodic recalls by an automatic system. The web system is in Italian, but a voice-over in English is also available. **At the end of FLIP project, 323 NAFLD patients were already using the web system**, continuously monitored for their participation into planned activities. **6-month results demonstrate that patients who complete the program lose approximately 3-4 kg body-weight** (>5% of baseline body weight in one third of cases), **liver enzymes (ALT notably) are reduced by 30% and normalized in approximately 40% of cases**. A limit of the web-based intervention is attrition, occurring at rates probably larger than observed during face-to-face contacts with therapists.

4. Potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

- **Prospective pan-European cohort of patients with NAFLD**

We have developed the largest multicentric European, transnational prospective cohort of 668 well-phenotyped NASH patients with standardised inclusion criteria.

This large cohort of patients included with comparable inclusion criteria and available biopsies from different clinical centers in Europe made possible to merge for the first time in Europe, standardized clinical, epidemiological and biological data and biological samples. Because of its size and the diversity of its epidemiological patterns of NAFLD, it has been a unique opportunity for the development and validation of new generation diagnostic tools, for performing genetic analysis and biomarker validation or identification.

The medical impact of this transnational database is considerable since being the largest database of hepatological NASH patients in Europe, it provides for the first time in Europe a large amount of information on the clinical condition of European NAFLD patients at baseline. It also validates simple clinical algorithms of risk stratification for the numerous patients exposed to the risk of NAFLD. From a scientific standpoint it achieves significant power when studying host factor - disease interaction.

In the future, this database will enhance scientific collaborations based on: serum samples (academic and industry), genetic markers and clinical and histological data (natural history), with academic or industry partners. It is a start and the beginning of a much larger cohort since our consortium is happy to include new clinicians' teams for expanding the biobank. It fills a big gap in terms of structured research efforts in NASH in Europe.

- **Consensual histological classification of NAFLD/NASH**

We developed novel validated and consensual scoring systems for liver biopsy interpretation that can be applied in the context of fatty liver disease for diagnosis system among the pathologists.

Previous semi-quantitative scoring systems in man have inherent weaknesses. Our group of pathology experts defined in a rigorous way the elementary histological lesions of NAFLD including the most difficult ones such as hepatocyte ballooning; it created a systematic score based on the Steatosis (S), Activity (A, ballooning and lobular inflammation) and Fibrosis (F) components that allows both a qualitative diagnosis in clinically relevant categories (NASH- a progressive condition and NAFL a rather stable condition) useful in clinical practice and epidemiology studies and a quantitative assessment of the severity of the disease useful for therapeutic trials. It validated this score in terms of reproducibility and interobserver variation among a group of expert pathologists (the FPG); this highly reproducible approach, will overcome many of the problems with other systems such as the NIH NAS score;
This new SAF score and algorithm can now be used in the larger population of patients with suspected NAFLD investigated in liver units. This new histological classification will be crucial in the assessment of cohorts such as the FLIP one and presents the benefit of producing consistency across the EU with respect diagnoses. Moreover, in the future, this will in turn improve large epidemiological studies.

- **Investigations on the epidemiology of NAFLD**

The conclusions of epidemiological analysis of the 3 cohorts from Bristol, Copenhagen and Italy, will help physicians in their follow up of populations and in the development of new prevention strategies. It led to a better knowledge of at risk populations in Europe.

- **Identification of genetic determinants of NAFLD**

This study has confirmed, for the first time, the genome-wide significance of PNPLA3 in NAFLD and has identified several other variants that have been validated in our second patient cohort as modifiers of NAFLD. These will inform future mechanistic studies that will improve understanding of pathogenesis and may lead to the identification of novel therapeutic targets. The current study, following additional mechanistic studies, will have wide-ranging impacts on therapeutic targeting of medication, individual risk stratification and tailored medical therapy.

- **Validation of improved diagnostic & prognostic markers for NAFLD**

Because FLIP used large cohorts of patients included with comparable inclusion criteria and available biopsies from different clinical centers in Europe, it was possible to merge for the first time in Europe, genetic with biomarkers of NASH/NAFLD. This was a unique opportunity for the development and validation of new generation diagnostic tools.

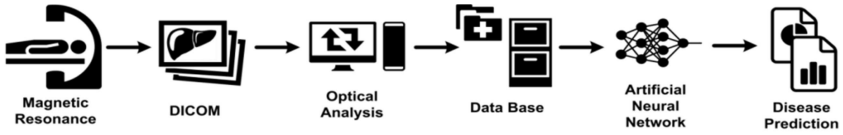
Diagnostic value of non-invasive biomarkers of liver injury in NASH and of their prognostic value in patients with hyperlipidemia, diabetes or morbid obesity : Fibrotest, SteatoTest and NASHTest

In preliminary work, we could show that these three biomarkers predict elementary histological lesions of NAFLD with the same accuracy as they predict the presence of the same lesions in patients with other causes of chronic liver diseases. We reviewed survival and liver related mortality as well as cardiovascular and liver-related events in on a cohort of 1909 patients with hyperlipidemia and a

cohort of 1133 patients with type2 diabetes. We demonstrated that no or early fibrosis as indicated by reduced baseline FibroTest values is predictive of survival without liver related complications.

Development of a new imaging method based on an MRI technology to allow a radiological, non-invasive prediction of steatohepatitis (NASHMRi) and of fibrosis (FibroMRi) in patients with NAFLD.

The FibroMRI technology has been tested on an estimation cohort of 32 NAFLD patients with different fibrosis stages. We defined an algorithm using neural network analysis with a success rate close to 80% for the prediction of fibrosis. We now will validate prospectively this algorithm in a larger validation cohort.



Scheme of FibroMRi and NASHMRi processes

Development of an NMR-based technique for visceral fat quantification.

We developed an NMR-based volumetric reconstruction imaging method for quantification of visceral fat independent from the abdominal subcutaneous fat. We obtained results from a first series of NAFLD patients and were able to establish correlations between visceral fat and intrahepatic fat.

- **Identification of clinical correlates and mechanisms of progression of NAFLD.**
  - **Metabolic factors and NAFLD**

Contribution of the omental adipose tissue to the pathogenesis and progression of NASH lesions

Our work opened new insights in the understanding of the contribution of the omental adipose tissue to the pathogenesis and progression of NASH lesions based on adipose tissue studies, microbiota, lipidomics analysis in liver disease in obesity. Our results can also have a strong impact on patient management since they can result in new markers for the follow up of the disease and help physicians in better tailoring treatment to patient profile, increasing chances of success.

We have demonstrated that there is an increase in CD68+ cell in visceral adipose tissue of NASH patients selected from a cohort of morbidly obese women undergoing bariatric surgery. After analyzing by multiplex the levels of a panel of cytokines in systemic and portal blood, we found that **CCL 22 can be used as a novel biomarker for NASH**. We are currently better defining the involvement of visceral adipose tissue in the release of this cytokine.
In addition, lipidomic analysis, performed by LC-MS in the same NAFLD patients, showed a particular lipid signature in systemic and portal blood involving phosphatidyl-glycerol (PG) species. Strikingly, the lipidomic profile in the adipose tissue secretion media was different from blood, strongly suggesting the involvement of the gut microbiota in the blood signature compared to adipose tissue.

We observed that, among 700 morbidly obese subjects, 16% had no liver lesions. These patients free of liver damage, in spite of being morbidly obese, display an increased total fat mass and a decreased

of the amount fibrosis in the subcutaneous adipose tissue. On the contrary, patients with severe liver fibrosis have also an increase in the adipose tissue fibrosis. These observations prompted us, in collaboration with Echosens, to create a new non-invasive tool, based on Fibroscan, to evaluate the degree of fibrosis in the adipose tissue. We have shown, for the first time, that **adipose tissue fibrosis can be measured and is linked to liver fibrosis** (assessed by Fibroscan). Adiposcan and Fibroscan could be used routinely in the clinic in order to better characterize morbidly obese patients and provide a tailored management.

We also confirmed in a larger cohort than the previously published one (Diabetes 2010), that **subcutaneous adipose tissue is predictive of weight loss induced by bypass surgery**, and that patients with higher level of adipose tissue fibrosis at the time of surgery were resistant to weight loss.

Role of hepatic triglyceride lipases in the progression to NASH

This subproject clarified the role of metabolic lipases such as ATGL (PNPLA2) and adiponutrin (PNPLA3), both belonging to the family of patatin-like lipases, as key enzymes determining lipid partitioning and lipid signalling in the progression of NASH. PNPLA3 not only determines hepatic triglycerides stores, but also FA saturation indices and mitochondrial energy expenditure, possibly explaining the association of genetic variants with progression towards NASH. Metabolic lipases such as ATGL (PNPLA2) determine not only the degree of steatosis, but also features required for disease progression such as inflammation, fibrosis and ER stress along the course of NASH. Part of these ATGL (PNPLA2) effects can be explained by providing endogenous FAs as ligands for the key metabolic transcription factor PPARalpha, for the first time linking lipid partitioning to lipid signaling.

Bile acids (BAs) and proteins involved in BA transport (BSEP) and signalling (FXR) were identified as key regulators of hepatic lipid metabolism and inflammation, however without impacting on metabolic lipases. Notably, therapeutic BAs such as UDCA facilitate lipid partitioning and thereby may counteract lipotoxicity by up regulation of the enzymes favouring desaturation of fatty acids/lipids.

Using high-field (3 and 7T) MR-spectroscopy we were able to non-invasively distinguish NAFLD from NASH and advanced fibrosis based on their 1H and 31P spectra reflecting FA/TG and membrane composition in addition to mitochondrial function (NADPH, ATP). Novel genetic and MRS biomarkers may allow non-invasive risk stratification for cost-effective follow-up and individualized treatment of a disease reaching the magnitude of an epidemic.

Metabolic lipases (PNPLA2 and PNPLA3) and BA transporters/receptors (BSEP and FXR) may represent prognostic markers (e.g. genetic variants) and potential therapeutic/pharmacological targets in NASH. In addition to a research tool, depending on technological advance, high field MR spectroscopy may become a non-invasive diagnostic tool for distinguishing NAFL from NASH.

Insulin resistance as a causal factor in the pathogenesis of NAFLD

Study of the complex relationship between insulin resistance (IR) and liver damage in NAFLD allowed us to provide a clear picture of the tissues and organs involved and of the relationship between histological features on one side and the degree of the metabolic derangements on the other.

Data from this WP provide useful tools that track the mechanisms of disease and can be used for non-invasive risk stratification, for cost-effective follow-up and individualized management of NAFLD/NASH. Based on our results, we therefore suggest that insulin resistance in the adipose tissue and in the muscle should be the main targets for therapeutic strategies in NAFLD either by tailored lifestyle intervention or by specific drug molecules. Moreover, abdominal circumference can be used as a screening tool for NAFLD in the general population, particularly in non obese, non diabetic subjects, were NAFLD is usually underestimated. Finally, fasting insulin levels can be used to screen NAFLD patients at risk for significant liver damage and to monitor the efficacy of therapy.

Our results therefore provide the pathophysiological rationale for insulin sensitizers specifically targeting the adipose tissue and the peripheral defect in insulin sensitivity. Hopefully, a metabolic improvement at this site will also lead to a regression of necroinflammatory lesions in NAFLD, and to prevention of fibrosis progression.

Relationship between metabolic handling of an oral fat load and the degree of liver damage in NAFLD

Data from this WP provide useful tools to be used for diet-based prevention, non-invasive risk stratification and individualized management of NAFLD/NASH.

Potential impact of our results rely in the knowledge of the mechanisms of fat absorption and metabolism and in the understanding of the deleterious effects of western diet, high in fat, on liver damage and metabolic complications of NAFLD including cardiovascular disease :

- A diet composition enriched in PUFA, linoleic acid in particular, and depleted in SFA could prevent the onset and the worsening of liver damage in NAFLD;
- The medium PUFA/SFA ratio can be used as a non-invasive marker of liver disease severity;
- Increased levels of fasting small dense LDL concentrations and oxidized LDL provide a link for increased risk of both liver damage and cardiovascular disease in NAFLD;
- ApoE genetic variants can predict the severity of liver disease in NAFLD.

▪ Inflammation factors and NAFLD

Role of microbiota induced by feeding a high fat diet on liver fibrogenesis

Our results indicate that dietary habits (e.g. a high-fat diet) result in an increased abundance of endotoxin-producing Gram-negative bacteria, which accelerate liver fibrogenesis. These observations indicate the possibility to manipulate intestinal microbiota to reduce progression of fibrogenesis in nonalcoholic steatohepatitis. In addition, the data suggest a possible differential role of the inflammasome system in the gut and in the liver, possibly leading to strategies differentially affecting this pathway for the modulation of injury and fibrogenesis associated with fatty liver.

Role and mechanisms of action of MCP-1

Our studies demonstrate that expression of MCP-1 plays a pivotal role in mediating activation of inflammatory pathways in experimental fatty liver. In the liver and in the adipose tissue, resident cells play a more relevant role compared to bone marrow-derived cells. Inflammatory changes parallel the disturbances in glucose handling and insulin resistance.

We have shown that a commonly used supplement has the ability to limit inflammation acting on the inflammasome, a potent and recently identified inflammatory pathway. Interaction of berberine with the P2X7 receptor may be exploited for the generation of new anti-inflammatory agents.

Our data indicate that resident cells are the most relevant contributors to MCP-1 secretion associated with metabolic disturbances induced by a high-fat diet. **Strategies dedicated to the modulation of MCP-1 expression specifically in these cells types are likely to be more successful in terms of amelioration of the metabolic and inflammatory picture associated with NAFLD.**

APE1/ref-1 as a novel modulator of inflammation and fibrogenesis in NASH

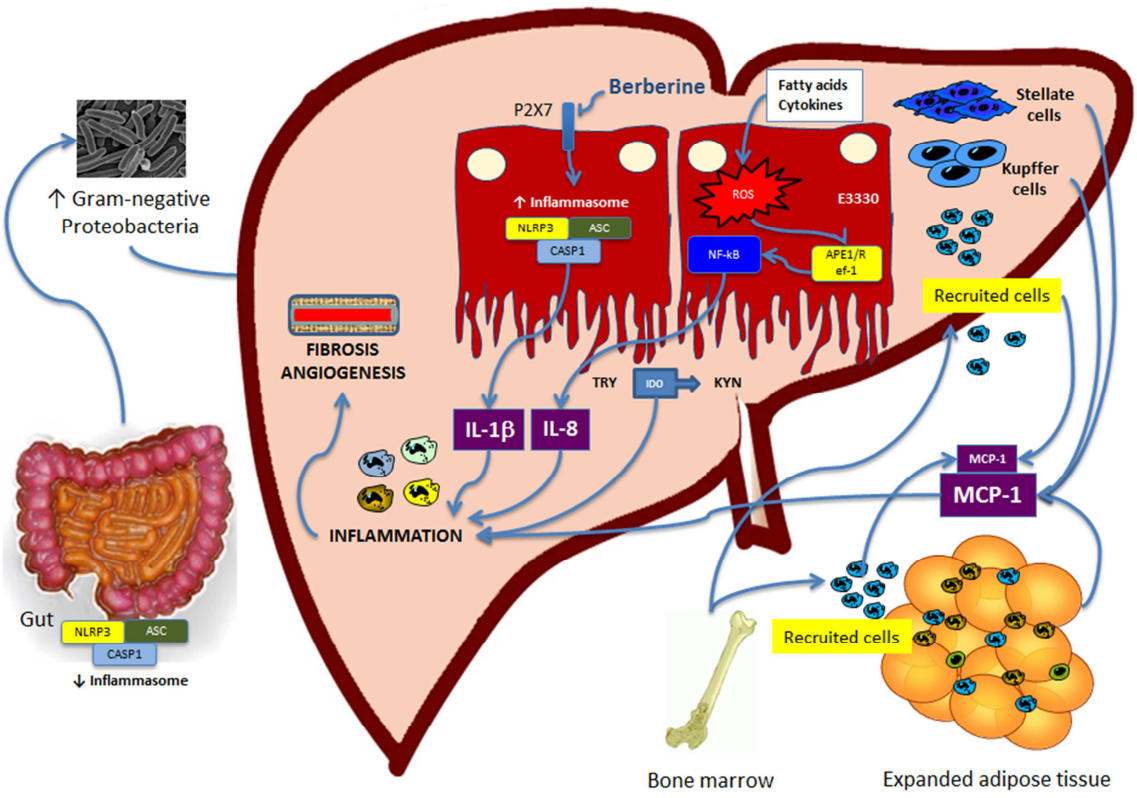
We identified **APE1/Ref-1 as a novel target potentially useful for the treatment of NASH.** In this set of experiments, we also developed a highly-reproducible model of cellular steatosis, to be used to

investigate interaction with fibrogenic cells. This *in vitro* model represents a valid tool for the early assessment of compounds potentially useful for the treatment of NAFLD.

Role of indoleamine dioxygenase (IDO)

We identified a potential novel modulator of inflammation, angiogenesis and fibrosis during steatohepatitis : indoleamine dioxygenase (IDO). Identification of IDO as a new factor implicated in inflammation and fibrosis will the field to experimentation of compounds targeting this pathway in the context of NASH, to block its progression.

The figure below summarizes the signaling pathways highlighted by our experiments; a new model of mecanisms of development/progression of NASH/NAFLD is thus arising from FLIP project.



Signaling pathways involved in NASH/NAFLD development and progression

- **Fibrosis factors and hepatic carcinogenesis**

Role of myofibroblast populations

Our work lead to the identification of a myofibroblast sub-population, as key cells in the vascular and architectural changes leading to cirrhosis. We therefore propose a new concept of cirrhosis formation whereby fibrosis expands from the portal tract in NAFLD. We provided new markers to investigate the functions of myofibroblast sub-populations in different organs affected in the cardiometabolic syndrome, including the liver, adipose tissue and cardio-vascular system. These markers may improve therapeutic targeting in NAFLD-related fibrosis and hepatocellular carcinoma.

Role of VDR

Evidence is provided for vitamin D supplementation and VDR targeting in NAFLD, including to maintain epithelial permeability and for an impact of high fat diet on changes in gut microbiota that promote inflammation.

Role of Kruppel-like Factor 6 (KLF6) in the pathogenesis of NASH progression and hepatocellular carcinoma (HCC)

We confirmed the central role of KLF-6 which becomes therefore a potential target for blocking disease progression.

SULF2, a KLF6 regulated target, was identified as a **biomarker** predicting poor prognosis in patients with HCC. SULF2 is therefore a candidate anti-cancer drug development target.

Creation of HCC registry

Moreover, this work package was very successful in establishing NAFLD/NASH as a bona fide cause of HCC and clarifying the clinical and carcinological presentation of this tumor. Increased awareness of this hepatic carcinological complication of NAFLD and data establishing associations with genetic markers (PNPLA3) will help refine proposals for targeted monitoring of subgroups of at-risk patients among the large number of exposed patients from the general population. Follow-up data are eagerly awaited as these will inform on therapeutic possibilities and their results in terms of tumor progression and survival.

- **Improved implementation of preventive strategies in NAFLD**

Results from studies on dietary and lifestyle habits of NAFLD patients are ready to be shared with actions undertaken by "The high level Group on Nutrition and Physical Activity" and "the Diet, Physical Activity and Health –Eu platform for action". A specific tool-kit for physicians and a web-based lifestyle intervention tool for patients were created to help, respectively; practitioners and patients identify behavioral and dietary risk factors of NAFLD and obstacles to change during implementation of lifestyle measures. These are ready to use instruments appropriate for clinical practice that fill a gap in our discipline and deal with aspects previously ignored or largely overlooked.

Influence of physical activity on the underlying disease leading to HCC

The experiments of acute and chronic exercise in mice fed a normal diet and a high-fat diet have provided evidence that exercise may reduce the tumorigenic potential in animals at risk to develop hepatocellular cancer. Although limited to well-defined experimental set-up, they provide a rationale to strengthen advice to physical activity in subjects at risk and may be used as background to carry out large scale epidemiological and intervention studies in NAFLD.

The studies on aerobic and resistance exercise provide clinical evidence of the beneficial effects of both types of exercise in NAFLD patients. In particular, the positive results of resistance exercise may be used to suggest this kind of physical activity to subjects who cannot perform aerobic training due to specific problems (severe obesity, joint involvement) limiting movements. These studies produced also complementary data on altered cardiac structure and function in NAFLD, as well data on autonomic neuropathy in keeping with the strict association between NAFLD and type 2 diabetes.

Our data indicate that aerobic exercise improves the metabolic milieu and reverses steatosis and show that it may be confidently used as background intervention in the prevention and treatment of NAFLD.

Improvements generated by resistance training occurred in the absence of change in body weight or diet. In summary, both aerobic and resistance training are effective, and the final decision as to which kind of exercise may be preferable in order to improve liver fat may be left to patients' preferences and available facilities.

A tool kit for patients' education and a web-based lifestyle intervention

The production of scientifically-sound manual to be used by physicians and other health personnel to educate NAFLD patients has spread outside the limits of the participating Units following dissemination activities both in Italy and outside Italy. Data on motivation to change and differences in perceived importance of healthy diet and habitual physical activity may be used to tailor specific programs to overcome barriers to lifestyle changes. The results led to develop a similar study in another population at risk, i.e., in type 2 diabetes. The web-based intervention has provided evidence of a positive effect of information technology as a tool to intervene in large section of the population. This evidence is of paramount importance considering the very large numbers of people at risk that cannot be addressed by conventional education. The web intervention also reduces barriers in young subjects at risk, limiting the need of fixed dates during working hours when job and time constraints limit attendance. The results have spread outside the boundaries of the FP7 program, due to an intense dissemination to other hepatology Units in Italy and is now also used directly by GPs as a support for their activity to subjects with overweight/obesity and type 2 diabetes, both in the presence and the absence of altered liver enzymes. The program was also proposed as an intervention strategy to manage patients with metabolic disorders to the Italian Ministry of Health and to a specific plan of Regione Emilia-Romagna.

5. Consortium and contact information

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For more details please see the project website: *http://www.flip-fp7.eu/*

6. Use and dissemination of foreground

58 Peer reviewed publications and 19 papers in proceedings of a conference have been published, among them 31 are on Open Access on the platform Open Aire. During the project, the consortium established special links with EASL (European Association for the Study of the Liver).