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The Steroltalk Consortium

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Steroltalk:
Functional genomics of complex regulatory networks from yeast to human: cross-talk of sterol homeostasis and drug metabolism

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http://www.steroltalk.net

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Table of contents

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2 Relevant links

- Consortium homepage: http://www.steroltalk.net
- STEROLTALK data portal: http://steroltalk.mf.uni-lj.si
- STEROLTALK microarray database: http://cfgbc.mf.uni-lj.si/base2
- STEROLTALK Express: http://www.ailab.si/ste/run

3 Project Execution

SUMMARY

The vision of STEROLTALK was to combine dedicated functional genomic tools, three model organisms and *in silico* modelling, for the discovery of new drug targets and novel chemical entities for treatment of hyperlipidemias, and to establish the drug-drug interactions. This has allowed STEROLTALK to importantly contribute to deciphering the multi-level response of cholesterol homeostasis to known drugs (statins) and candidate drugs (LK-compounds). Most of research has been performed in human primary hepatocytes and in wild type or nuclear receptor knockout mouse livers, and in recombinant yeast strains that express key human enzymes of cholesterol synthesis. In addition to commercially available tools we have developed original STEROLTALK tools for functional genomic studies of the hypolipidemic drugs . Tools include a dedicated STEROLTALK microarray in the human and mouse version, that has been used successfully in drug-perturbation studies of the human primary hepatocytes and in mouse experiments. The STEROLTALK antibody set includes over 20 evaluated antibodies for the human drug metabolizing cytochromes P450 (CYPs) and anti-peptide antibodies against enzymes of the cholesterol synthesis, majority of which are novel and are not available commercially. The STEROLTALK recombinant yeast strains that overexpress human enzymes of the cholesterol synthesis pathway were not only proven to represent original hypolipidemic drug screening tools, but are also a resource for the recombinant human cholesterogenic enzymes that can be used for kinetic studies or applied as epitopes for the new antibody production. Analytical (microarray and RT-PCR results) and sterol metabolome data and a limited set of the available western analyses results have been joined with the literature data to build the *in silico* model of cholesterol synthesis. This dynamical model was able to reproduce the experimentally verified action of statins, the well known drugs of cholesterol synthesis, as well as the two novel potential drugs from the LK series of Lek Pharmaceuticals. The STEROLTALK goal to build an *in silico* model that would predict the action of the novel hypolipidemic drugs, has thus fully been reached. Additionally, the Byesian approach to construction of gene regulatory networks has lead to the prediction of another transcriptional regulator of the cholesterol synthesis, which has been proven experimentally. Furthermore, proteomic tools (2D gel electrophoresis and nLC-MS) have been established, optimised and successfully used for the analysis of human hepatocytes. In addition to already known players, proteins not yet known to be involved in STEROLTALK relevant regulation processes were found to be differentially regulated. Last but not least, the STEROLTALK database served as a share point for the exchange of experimental protocols and samples with unique Steroltalk coded signatures. The database served as well as a repository for the transcriptome and RT-PCR data, applying the Base 2.7.2. software that includes the MIAME standard of the minimal required information about a microarray experiment.

It is important to note that the formal end of the EU funding of the STEROLTALK does not mean an end to the developed STEROLTALK tools, models and samples. University of Ljubljana (P2 of Steroltalk), where the scientific co-ordination took place, will continue to

maintain and upgrade the STEROLTALK database in collaboration with Crea, d.o.o., P10 of this project. The database will remain available to the STEROLTALK partners without additional cost for one year and will be continued afterwards with the shared cost contribution. Additionaly, the model of the successful sharepoint within an international consortium, for experiment, protocol and sample exchange, will be used as a template in planning research under the current and future FP7 systems biology calls. Also other STEROLTALK tools will remain alive in further research – the idea of a dedicated microarray that, in combination with commercial pan-genomic arrays, leads to a much quicker hypothesis formulation; the collection of STEROLTALK antibodies and the heterologous expression of STEROLTALK proteins in the yeast, will be very helpful in further research and can as well find commercial application. Additionally, research samples, such as mouse livers, human and mouse RNAs and other isolates, remain available for additional analyses within the broader scientific community. These valuable research samples from well controlled STEROLTALK experiments can contribute to a quicker solving of the questions regarding the cholesterol synthesis and drug metabolism networks, that remained un-answered after the 3-year STEROLTALK period.

The reached Steroltalk project objectives in relation to the state of the art and the impact of the project on the industry and research sectors

The STEROLTALK project objectives were defined as follows:

Objective 1:
To develop original Steroltalk functional genomics tools which will complement commercial tools, to enable the realization of Objectives 2 and 3. Original tools include:

- A dedicated Steroltalk microarray;
- A structured Steroltalk database and in silico models with a predictive power;
- A set of Steroltalk antibodies;
- Humanised yeast for the late portion of cholesterol biosynthesis.

Objective 2:
To experimentally determine the cross-talk between cholesterol homeostasis and drug metabolism in human primary hepatocytes and in livers of normal, hyperlipidemic and nuclear receptor-knockout mice, at the level of transcriptome, proteome and metabolome and to include these data in the in silico Steroltalk model.

Objective 3:
To apply the Steroltalk tools and experimental data together with mathematical models to determine novel correlations and resolve unknown molecular mechanisms of the cholesterol homeostasis/drug metabolism network. This will aid in definition of new drug targets (effects and potential side-effects) of known and novel chemical entities and may lead to novel therapeutic strategies.

The objectives of STEROLTALK have been fulfilled and were in line with the proposed WP flow (Fig. 1), as well as with the scope of the available 3-year time.

Objective 1:
As mentioned above, all promised STEROLTALK tools have been developed and experimentally explored to reach the STEROLTALK biological results and conclusions. Majority of tools have proven to be useful for further research and development beyond the EU FP6 programme. The STEROLTALK consortium agreement regarding the precise plans for the use of the STEROLTALK tools after the completion of the FP6 project is described at the end of this session.

Objective 2:
The major breakthroughs in the experimental determination of the cross-talk of cholesterol homeostasis and drug metabolism were at the level of transcriptome. Here the dedicated STEROLTALK microarray allowed realization of over 200 microarray experiments. In combination with some pan-genomic arrays, the STEROLTALK transcriptome experiments revealed novel mechanisms of the nuclear receptor-mediated regulations of this network, uncovered the actions of LK-series of novel potential cholesterol lowering drugs and proposed some deregulated pathways that can explain the side effects of the well known cholesterol lowering drugs (statins). Before reaching the final conclusions, the relative transcriptome measurements have been, for the key genes, upgraded by quantitative RT-PCR. At the protein level the quantification of the STEROLTALK proteins was done using Western blot analysis as classical approach and 2D gel electrophoresis and nLC-MS as state-of the art analytical techniques. Probes of the developed STEROLTALK antibody set were successfully used in the Western blot experiments to get quantitative data of STEROLTALK relevant proteins. The 2D gel electrophoresis was established, optimised and used for the analysis of a restricted set of STEROLTALK human hepatocytes. While 2D gel electrophoresis delivers data about the soluble cytosolic proteins of the samples, nLC-MS was, additionally to the original project set-up, included as promising and innovative tool to get quantitative data of membrane proteins of the microsomal fraction. Both methods are by themselves not able to deliver high-throughput proteomic data but opened and fulfilled the expectations to get data about proteins not yet know to be affected by statins. These proteins may represent starting points for further studies because they may be involved in STEROLTALK relevant regulation processes. The STEROLTALK metabolome measurements were advanced in its quantitative nature. A novel GS-MS method for separation and quantification of ten sterols, all cholesterol synthesis intermediates, has been developed and successfully used within STEROLTALK and beyond. Quantification of the bile acid pathway in hepatocyte cells and in media has also been performed. Some of the sterol metabolite data have already been combined and correlated to the transcriptome data for the use in *in silico* modelling. The majority of sterol metabolome data remain available for the future bioinformatic analyses. Another set of quantitative data came from the metabolic capacity tests, where the enzyme activities of the major cytochrome P450 metabolizing enzymes (CYPs) have been evaluated in each human primary hepatocyte culture, before and after the STEROLTALK drug perturbation experiments. Two siRNA trials have been performed to mimick at the genome level the effect of the two novel potential

cholesterol lowering drugs. Complementary transcription regulation studies, especially within the area of the drug-dependent nuclear receptor-mediated disturbances of the cholesterol homeostasis, have also been performed, to test the proposed hypotheses.

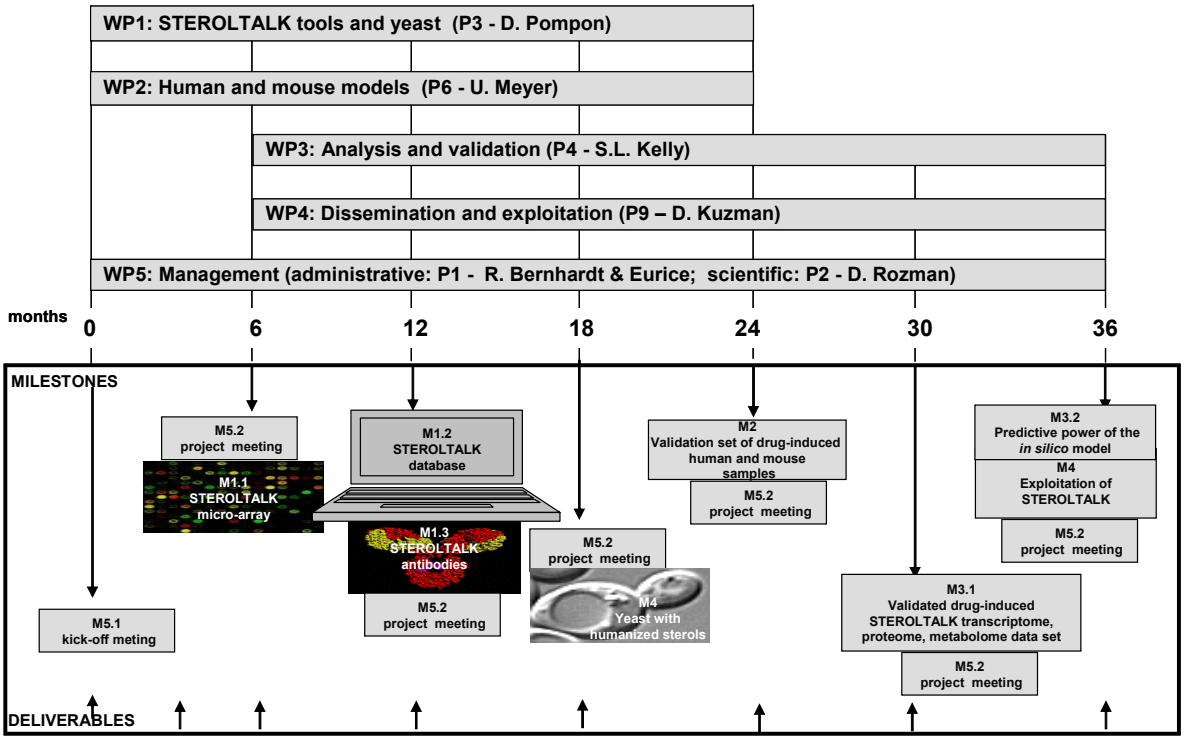

Figure 1: The workpackage flow of the Steroltalk project.

The major biological conclusions from the STEROLTALK experiments are listed bellow.

The experiments with the wild type mouse hyperlipidemia models revealed:

1. Chronic treatment with poloxamer P-407 triggers a concerted modulation of hepatic genes involved in multiple steps of atherogenesis. (Kuzman et al., Acta Chimica Slovenica, accepted)
2. Downregulation of the hepatic cholesterol synthesis is a hallmark of mouse hyperlipidemia models. Statins and fenofibrate provoke changes in the hepatic transcriptome after more than 24h treatment (Rozman D. et al., in preparation: The Steroltalk mouse models to study hyperlipidemia and its treatments)
3. Nuclear receptor CAR regulates the mouse liver cholesterol homeostasis in a SREBP2-indenedent manner (Rezen T. et al., submitted to Hepatology).
4. High-fat medium and circadian transcription factors contribute to the regulation of cholesterogenic Cyp51 and Hmgcr genes in mouse embryonic fibroblasts. Fink et al., *Acta chim. slov.*. 2008, 55: 85-92).

5. cAMP responsive element modulator CREM modulates the circadian expression of CYP51, HMGCR, and cholesterogenesis in the liver. (Acimovic et al., *Biochem. Biophys. Res. commun.*, 2008, 376: 206-210)

The experiments with the nuclear receptor knockout mouse models revealed:

1. CAR and PXR activators (TCPOBOP and PCN) and pan-inducers (phenobarbital) affect numerous genes involved in lipid homeostasis (Tamasi et al., submitted, Rezen et al., 2007). Numerous new hypotheses on the regulation of cholesterol homeostasis have been derived from these experiments.
2. Diets high in cholesterol (1 – 2 %) reduce drug metabolism and the induction of cytochromes P450 via LXR and SREBP1 (Gnerre et al., 2005; Roth et al., 2008).
3. Bile acids in physiological concentrations induce cytochromes P450 (e.g. CYP3A4) via FXR and induction of PXR (Jung et al., 2006).
4. Activation of CAR and PXR acutely lowers triglycerides in liver and plasma by activating Insig-1 (Roth et al., 2008).
5. Bile acids induce ALAS-1, the first enzyme of heme synthesis, by activating FXR (Peyer et al., 2007).

Experiments with the human primary hepatocytes resulted in following conclusions:

Changes in human primary hepatocytes induced by atorvastatin and rosuvastatin at 24 hours are extensive. Over 1000 differentially expressed genes were detected and many biological pathways are affected. There are some similarities between the two statins, however, more dissimilarities are observed. For instance one, already shown by the Steroltalk microarrays, is that atorvastatin is a much stronger activator of drug metabolism than rosuvastatin. However, to fully elucidate the affect of statin treatment and also possible explanation of certain side-effects more knowledge driven data analysis by data biocurators is necessary. It is important to note that there are no data available in the literature showing the wide genome effect of statins on the hepatic transcriptome. Atorvastatin is a strong activator of PXR and by this some effects on gene expression could be an indirect effect of PXR activation. A comparison with rifampicin, a classical PXR activator, would be necessary. Rosuvastatin also induced some drug metabolizing genes, but not to such extent as atorvastatin. Important difference between both statins is in time-dependent changes. When rosuvastatin gene expression peak is at 24 hours, atorvastatin effect is still rising at 48 hours. Both have other important similarities and differences. Both upregulate cholesterol biosynthesis and *INSIG1*; gluconeogenesis; fatty acid synthesis; lipid plasma transport. However this effects could result from different mechanisms by which statins exert their effect since they differently affect expression of transcription regulators *SREBF1, SHP, LRH-1*.

Important as well is upregulation of gluconeogenesis at 12h by both statins, under normal growth conditions when hepatocytes do not enter starvation. Further research is required to evaluate whether upregulation of gluconeogenesis might require upregulation of protein degradation which is connected to some serious side effects of statins, including myopathy and rabdomiolysis.

LK935 is metabolized fast in human primary hepatocytes therefore it is difficult to talk about its effect on gene expression. LK980 on the other hand exhibits biphasic response in gene

expression. To explain such response we would have to know how LK980 is metabolized. It could be possible that we see an effect of two compounds, for instance at the beginning we could see the effect of the LK980 itself, which is later metabolized so at 48 hours we see the effect of its metabolite. However, such hypotheses are impossible to form until we know the metabolism rate of LK980. If comparing LK980 expression profile to stains, we see an interesting similarity in up-regulation of the *CYP4F* family.

Transcriptome data are still in process of being analyzed and biologically interpreted. The whole genome transcriptome data comparison to the proteome and the sterol metabolome data is also in progress. It is expected that at least two additional high rating publications will result from this part of the Steroltalk collaborative work. The use of the human primary hepatocyte models treated by statins and novel cholesterol lowering drugs have so far resulted in 4 publications that are either accepted or in final drafts, and in 2 international patents:

1. Novel cholesterol biosynthesis inhibitors targeting the human lanosterol 14[alpha]-demethylase (CYP51). (Korosec T et al., *Bioorg. Med. Chem.*. [Print ed.], 2008, vol. 16, no. 1, str. 209-22).
2. Expression of microsomal lanosterol 14alpha-demethylase (CYP51) in an engineered soluble monomeric form. Seliskar M, Kosir R, Rozman D., (Biochem Biophys Res Commun. 2008 Jul 11;371:855-9).
3. Drug-interaction potential of LK-935, the novel non-statin type cholesterol lowering agent. (Monostory K. et al., *Drug Metabolism and disposition,* 2008, Oct 29. [Epub ahead of print]).
4. *Novel derivates of pyridylethanol (phenylethyl) amines as inhibitors of cholesterol biosynthesis, processes for their preparation, and pharmaceutical compositions containing them*. (Rode et al., 11.08.2006. Muenchen: European patent office, 2006).
5. *Heterocyclic compounds : WO2007/073935*(U. Urleb et al., World Intellectual Property Organization, 2007; Geneve).
6. Novel Piperazines – Distal Inhibitors of Cholesterol Biosynthesis. 14-reductase main target? (J. Acimovic et al., in submission to *Bioorganic and Medicinal Chemistry)*.

Objective 3

The application of the Steroltalk tools and experimental data together with mathematical models has been used successfully to resolve unknown molecular mechanisms of the cholesterol homeostasis/drug metabolism network. This resulted in definition of new drug targets, the effects and potential side-effects of novel potential cholesterol lowering drugs as well as of known cholesterol synthesis inhibitors statins.

The humanized STEROLTALK yeast strains that have been produced by recombinant yeast engineering, express one or several of the human enzymes of cholesterol synthesis. They do not accumulate the natural yeast ergosterol, but instead produce cholesterol or intermediates of its synthesis. The best models for drug screening were found to be single- or multiple-substituted yeast strains producing cholesterol as the end product. We validated the

humanized yeast CYP51 strain with respect to gene expression, protein localization, growth characteristics, and sterol content. The strain showed up to >1,000-fold-reduced susceptibility to the orally active azole drugs, while the topical agents showed no difference. This strain provides a useful tool for the rapid and high-throughput initial specificity testing of new drugs designed to inhibit fungal CYP51. This is an *in vivo* system that allows direct comparisons of the specificities of the drugs and could also be used to further investigate inhibitors of CYP51 not only for antifungals but also for hypolipidemic therapies. For example, LK-935 as a novel potential cholesterol lowering drug, inhibits the human CYP51 enzyme.

The recombinant humanized cholesterol producing yeast strains with multiple humanizations allow a global view of the impact of drugs or expression polymorphism on the sterol metabolome. At this level humanized yeast is both complementary with modeling and a unique tool to define kinetic parameters of human enzymes required for *in silico* model refinement. The development of a proof of concepts for the use of humanized strains as drug screening tool has only been initiated during the project course, but has a much larger exploration potential. A significant differential response at the level of growth rates and sterol compositions has been observed upon treatment with different cholesterol lowering drugs. However, further developments are needed to establish a robust protocol of drug evaluation for cholesterol producing strains which can be used in high throughput conditions.

Additional advantages of the humanized yeast model is, as mentioned, to provide reference material for proteome research, such as individually expressed human proteins and sterol intermediates of human cholesterol synthesis.

The STEROLTALK humanized recombinant yeasts have so far revealed one SCI publication. Patenting of the strains is being discussed before other data will be released as publications.

1. Parker JE, Merkamm M, Manning NJ, Pompon D, Kelly SL, Kelly DE. Differential azole antifungal efficacies contrasted using a Saccharomyces cerevisiae strain humanized for sterol 14 alpha-demethylase at the homologous locus.

Steroltalk mathematical models

Construction of *in silico* models with predictive power was an important objective of the STEROLTALK project. Models have the power to act first as an integrative framework for the STEROLTALK data. They also have qualitative predictive power. The obvious limitation was the difficulty to build a quantitative model for a complex network with a limited number of experiments. This represents a general drawback that became obvious to the scientific community during the course of the Steroltalk project and is still a general limitation in system biology. Types of experiments required to build a quantitative model frequently differ from the experiments required to answer specific biological questions of an immediate interest. To capture the dynamic processes of a regulatory network, the rapidly changing parameters have to be determined repeatedly and quantitatively, which poses major challenges to the experiments used for model building.

Validation of dynamical models was performed on data accumulated during the STEROLTALK project, on the previous data for the effect of statins and LK compounds in human immortal hepatocytes and from the literature. The humanized recombinant yeast data (sterol measurements on the 11 LK-treated humanized recombinant yeast strains) are being incorporated in the current models. Similarly is true for the protein data from drug-treated human hepatocytes

To come to the conclusions bellow, mathematical models of dynamic character were chosen, to match the character of the modelled process. However, they were simplified as much as possible, since important entities were measured often only at three time points which is not sufficient for studying the kinetics.
The currently best validated are models of human hepatocytes and the human immortal hepatocyte HepG2 cells. The model was able to predict two regulation mechanisms that were confirmed in the literature (degradation of HMGCR by lanosterol and 7-dehydrocholesterol) while a regulation of lanosterol metabolising enzyme was indicated, but not found in the literature. According to the model, the cholesterol lowering effect of LK compounds occurs through indirect degradation of HMGCR. Blocking of HMGCR is in the case of LK compounds not as strong as in the case of statins, however, side effects can be caused by the accumulation of intermediates. There is also a significant difference in cholesterol biosynthesis between human hepatocytes and HepG2 cells indicated by the model. Human hepatocytes seem to have less effective degradation effect of 7-dehydrocholesterol on HMGCR than HepG2 cells.

In parallel with dynamic models, a tool for automatic generation of metabolic networks was developed. The tool can recreate a metabolic network of all theoretically possible metabolites given the structure of the first substrate and functionality of the involved enzymes. The tool is valuable to get information which metabolites can be generated in order to identify escape routes in metabolic pathways and for designing proper measurement protocols that would enable reliable measurement of all the involved metabolites. It can also be used for identification of specific enzyme functionality. A variety of outputs were achieved in collaboration in the STEROLTALK project and the modelling generated supported hypothesis generation that was subject to experimentation around the ability of the network of reactions to bypass cholesterol. Future refinement is ongoing after the formal end of STEROLTALK.

It has long been demonstrated that the level of cholesterol in cells regulates the cholesterol biosynthesis through SREBF transcription factors, but lately it has been shown that other factors are also important. To study the system we combined mathematical modeling and simulation with Bayesian network inference. We constructed a mathematical model of cholesterol biosynthesis and studied its properties through simulation. We measured transcriptional changes of cholesterogenic genes using the Steroltalk microarray and treated human hepatocyte samples. We employed Bayesian network inference to identify gene-to-gene interactions from both microarray measurements and simulated data. The inferred Bayesian networks show that expression of cholesterogenic genes can be predicted from

expression of 4 key genes, one of them being *SREBF2*. Networks also indicate a strong interaction between *SREBF2* and *CYP51A1*, but not between *SREBF2* and *HMGCR*, the rate-limiting enzyme, whose expression seems to be regulated by other factor(s). Simulations exposed that a large number of perturbations of the system is critical for identification of gene-to-gene interactions, and that measurement noise and differences between human individuals pose a serious problem for their automatic inference from microarray data

The STEROLTALK modelling efforts have so far resulted in following publications:

1. Towards identification of gene interaction networks of human cholesterol biosynthesis. Juvan et al., *Acta Chim. Slov.* 2008, 55: 96-407.
2. Object oriented modelling of metabolic pathways Belic A. et al., *5th MATHMOD : proceedings* Vienna: Argesim, 2006, 2: 8.
3. Physiology based model of cholesterol metabolism and its interactions with xenobiotics. Belic A et al., *Modelle und Simulationen in Medizin und Gesundheitsmanagement*. Wien: [S. n.], 2007, 4 str.
4. Automatic generation of cholesterol biosynthesis metabolic network. (Belic A et al., in review in *Journal of Theoretical Biology*.

4 Dissemination and Use

The Steroltalk consortium members did not only participate in a large umber of meetings and conferences to disseminate knowlegde generated and inform about project progress, but were particulary active also organizing and co-organizing a number of large international meetings:

- The MDO Meeting in Budapest (2006) was hosted and co-organized by HAS Katalin Monostory.
- The Swansea meeting (2006) was hosted by UWS D. and S. Kelly, with USAAR Riita Bernhardt being a Scientific Board member.
- The P450 Meeting in Bled was organized by UL_MF Damjana Rozman (USAAR Rita Bernhard being part of the International Scientific Board)
- Rita Bernhardt was furthermore a member of the International Scientific Advisory Board at the Moscow meeting 2008.

The 3rd FEBS Advanced Course "Cytochrome P450 systems: from structure to application" (23.-28.9.2008 in Kranjska Gora) was organized by USAAR Rita Bernhardt, together with University of Ljubljana. Additional Steroltalk partners involved were BIOZ Urs Meyer, UWS Steve Kelly, UL_MF Damjana Rozman.

Especially in the last project year, also a large number of publications derived from the project.

The following section will summarize the developed tools and materials and their further use after the project end:

Destiny of the Steroltalk tools, materials and samples after the formal completion of the project

As mentioned at the beginning, the STEROLTALK consortium has produced many valuable tools and materials that remain interesting for the research of the cholesterol homeostasis and drug metabolism network beyond the FP6 framework scheme. The Steroltalk consortium has decided for the status of the Steroltalk tools as listed below:

4.1 Steroltalk database

The data portal http://steroltalk.mf.uni-lj.si will remain accessible through the portal of the Centre for Functional Genomics and Bio-Chips, Faculty of Medicine, University of Ljubljana http://cfgbc.mf.uni-lj.si/. CFGBC takes the finantial responsibility for the 1st year of database maintenance and support. Steroltalk partners that will remain interested for this portal in 1 year after the completion of the Steroltalk project, will have to financially participate afterwards.

4.2 Steroltalk microarrays

The arrays can after Steroltalk "belong" to the Ljubljana group. CNRS – remains capable to spot slides, after UL is able to produce them good, they are free to do it.

4.3 Steroltalk antibody set

No antibodies are left after the project. What remains is a table of antibodies, their specificity, the peptide used for immunization and the yeast expression vector for the recombinant protein.

4.4 Steroltalk antibody protein chips

Several companies are interested in these chips. CNRS and USW are free to search for companies to produce a chip or use recombinant strategies to produce antibodies in a reproducible manner.

4.5 Steroltalk yeast strains

The yeast strains will remain of interest for some consortium partners as a source of human cholesterogenic proteins and human sterol synthesis intermediates. CNRS and USW are free to search for companies that would be interested to use these humanized recombinant yeast strains in anti-cholesterol or anti-fungal drug screening or development.

4.6 LK compounds

LK compounds will remain available after the end of the project until they are on stock. No additional synthesis will take place at Lek. LK-935 and LK-980 are available in 500 mg quantities, other LKs at lower quantities.

4.7 Toolbox for matlab

The toolbox will be applied further for modeling studies. UL is free to investigate possibilities for its commercialization.

4.8 Knockout mouse models

Single and double knockout mice that have been used at Biozemtrum, University of Basel, are stored at the site in an embryo stage with frozen sperm. Living animals remain available at Rolf Meyers lab in Freiburg. Some of the used knockout (Pxr and Car) are available commercially.