THALAMOSS Report Summary

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Final Report Summary - THALAMOSS (THALAssaemia MOdular Stratification System for personalized therapy of beta-thalassemia)

Executive Summary:
Description of the project context and main objectives. The THALAMOSS Project was aimed to provide comprehensive information on the relationship of genotype and phenotype in β-thalassemia beyond the effects of primary mutations and major modifiers of basic disease severity. In this respect, the disparity of studies on isolated aspects of the disease and the prevalence of a multitude of complex in vitro models for the analysis of therapeutic efficiency preclude a comprehensive assessment of interactions between genotype, transcriptome, proteome, induction of fetal hemoglobin (HbF) and gene-therapeutic expression of exogenous β-globin. For the first time THALAMOSS has compared and analyzed jointly data from independent international medical centers on patient characteristics and pertaining molecular data, potential therapeutic responses and critical aspects of medical regimens. Correlation of existing medical regimens, including blood transfusion (frequency and onset) splenectomy and chelation therapy, with other parameters under study and with medical indicators of the quality of life of patients will guide future patient-specific predictions of favorable treatments and disease progression. The anticipated complexity of combinations of different primary mutations with modifiers and novel markers necessitated a high number of patient samples, beyond the scale of any analyses performed to date, in order to allow a stratification of patient subgroups.

Main R&T results/foregrounds. The total number of recruited patients has been more than 900. The cellular β-THAL Biobank is constituted of more than 1340 vials from 213 patients. The most represented genotypes are β039/β039 (102 patients, 755 cryovials), β+IVSI-6/β+IVSI-110 (35 patients, 59 cryovials), β039/β+IVSI-110 (23 patients, 160 cryovials), β+IVSI-110/β+IVSI-110 (13 patients, 120 cryovials). Genomic DNA samples from more than 100 β-thalassemia patients have been used for identification of β -globin gene mutations, polymorphisms of the β-globin genes, the XmnI polymorphism, polymorphisms of the BCL11A, HB S1L-MYB and KLF1 loci (which are disease modifiers through their role in fetal γ-globin expression). DNA and RNA samples from these patients are stored for further genomic and transcriptomic analyses by the THALAMOSS consortium. Proteomic and transcriptomic studies allowed identification of genes differentially expressed depending on clinical parameters or HbF production; interestingly we found that transcriptomic pattern are also related to the response of HbF inducers. Novel biochemical and molecular biology markers in β-thalassemia were identified, among which: raptor, LYAR, KLF1, MAP3K5 gene, ASH1L, ZF-Ldb1. Novel experimental systems were developed. Functional studies demonstrated novel roles of microRNAs (miR-210) and transcription factors (LYAR) in HbF expression. Novel HbF inducers and stop codon correctors were identified. Novel gene therapy vectors were developed and gene editing approached proposed. At the end of the project, the cohort in the THALAMOSS Data Management Platform included 663 patients and one cell line, 42 RNA-seq analyses and 9x6 proteomic analyses, and sequencing data coming from cell lines (overall size of omics data totals to 204GB). Novel diagnostic procedures were developed useful for non invasive prenatal diagnosis.

Impact. The total number of peer-reviewed publications is 68. One patent is under formal presentation. A first clinical trial based on Sirolimus (rapamycin) is proposed. The THALAMOSS results are expected to be the basis for the development of personalized therapy and precision medicine.
1.1. BASIC INFORMATION ON β-THALASSEMIAS. The β-thalassemias are hereditary diseases caused by more than 350 mutations of the adult β-globin gene, leading to low or absent production of adult hemoglobin (HbA). Together with sickle cell disease (SCD), thalassemia syndromes are among the most impactful diseases in developing countries, in which the lack of genetic counseling and prenatal diagnosis have contributed to the maintenance of a very high frequency in the population. This conclusion is sustained by the worldwide presence of more than 270 million carriers of hemoglobinopathies, of which 80 million are carriers of β-thalassemia. Recent reports indicate that between 300,000 and 400,000 babies are born with a serious hemoglobin disorder each year (of which 23,000 with β-thalassemia major) and that up to 90% of these births occur in low- or middle-income countries. The management of β-thalassemia patients is mostly based on blood transfusion, chelation therapy and, alternatively, on bone marrow transplantation. Recently, novel therapeutic options have been explored, such as gene therapy, gene editing and fetal hemoglobin (HbF) induction. In consideration of the fact that β-thalassemias are characterized by several different gene mutations, they can be considered for effective personalized therapy on the road of precision medicine.

1.2. STRATEGIC OBJECTIVE ADDRESSED. The THALAMOSS Project was aimed to provide comprehensive information on the relationship of genotype and phenotype in β-thalassemia beyond the effects of primary mutations and major modifiers of basic disease severity. In this respect, the disparity of studies on isolated aspects of the disease and the prevalence of a multitude of complex in vitro models for the analysis of therapeutic efficiency preclude a comprehensive assessment of interactions between genotype, transcriptome, proteome, induction of fetal hemoglobin and gene-therapeutic expression of exogenous β-globin. For the first time THALAMOSS has compared and analyzed jointly data from independent international medical centers on patient characteristics and pertaining molecular data, potential therapeutic responses and critical aspects of medical regimens. Correlation of extant medical regimens, including blood transfusion (frequency and onset) splenectomy and chelation therapy, with other parameters under study and with medical indicators of the quality of life of patients will guide future patient-specific predictions of favourable treatments and disease progression. The anticipated complexity of combinations of different primary mutations with modifiers and novel markers necessitated a high number of patient samples, beyond the scale of any analyses performed to date, in order to allow a meaningful stratification of patient subgroups.

1.3. THE NETWORK. The THALAMOSS Network is constituted by University of Ferrara (UNIFE, Italy), The Cyprus Foundation for Muscular Dystrophy Research (CING, Cyprus), Erasmus Universitair Medisch Centrum Rotterdam (EMC, The Netherlands), Biomedical Research Foundation, Academy of Athens (BRFAA, Greece), Weill Cornell Medical College, WCMC (CU, USA), The Children’s Hospital of Philadelphia, Philadelphia (CHOP, USA), Masaryk University (MU, Czech Republic), University of Cagliari (UNICA, Italy), King’s College Hospital (KCL, UK), Laiko General Hospital (LGHA, Greece), NOVAMECHANICS Ltd., Cyprus, Harbour Antibodies BV (The Netherlands) and IRBM Science Park (Italy). The SME BIOCEP, Israel, was also involved the first period of research activity.

1.4. SPECIFIC OBJECTIVES. The objectives of THALAMOSS can be summarized as follows: Development of a universal set of techniques for the stratification of β-thalassemia patients into treatment subgroups for (a) onset and frequency of blood transfusions, (b) choice of iron chelation, (c) application of chemical inducers of endogenous foetal haemoglobin (HbF), (d) prospective efficacy of gene-therapy intervention with globin-expressing vectors. A combination of these modular techniques into an integrated approach would allow more accurate genetic counseling of carrier couples at risk and comprehensive guidance of therapeutic interventions for β-thalassemia patients.

The THALAMOSS strategy was based on the acquisition of peripheral blood samples from patients (n≥500) and controls (n≥50). The performance of genetic (SNP) analysis of nucleated blood cells and the employment of proteomics and functional genomics analyses allowed a subdivision of patients into sub-classes. Establishment of a standard cell culture protocol for
erythroid precursor cells (ErPCs) and of bio banks allowed the determination of the activity of HbF inducers, as well as the correction of the molecular defect with standard and customized (mutation-specific or HbF-inducing) lentiviral vectors. Medical records and cell-culture analyses have been correlated with genetic, proteomics and functional genomics findings. Assessments included the combination of known disease modifiers, such as BCL11A, KLF1, SOX6 and HPFH (hereditary persistence of fetal hemoglobin) mutations, and determination of novel SNP, mRNA and protein markers, towards (a) more reliable prediction of expected phenotypes of novel subclasses for genetic counseling, (b) optimized treatment with conventional therapies and (c) an establishment of personalized-medicine therapies for the novel approaches of HbF induction and lentiviral gene therapy (GT).

The specific aims of the THALAMOSS Project have been achieved by the development of novel methods for associating variation in genomic data with phenotypic variation, leveraging the rich content of public annotation databases and the highly specialized knowledge of partners in the network. THALAMOSS combined cutting-edge computing technology with optimized algorithms to mine the unique datasets provided through the proposed project for biologically and medically relevant patterns that can be reliably associated with specific patient groups, treatment response and disease progression. In addition to stratification of patient samples for their molecular properties, THALAMOSS has also analyzed responsiveness to advanced therapeutic approaches to classify patient samples, analyzing this responsiveness both as a consequence of molecular properties and as a determinant of the success of novel therapies. At present, the most promising novel approaches to β-thalassemia treatment are the application of chemical inducers of endogenous fetal hemoglobin (HbF) and transduction of hematopoietic precursor cells with lentiviral vectors expressing exogenous β-globin. Accordingly, the proposed project was aimed at standardize a high-throughput in vitro differentiation protocol of patient-derived erythroid precursor cells and use it to test established inducers of HbF and established β-globin-expressing lentiviral vectors for their therapeutic efficacy and cytotoxicity in a large number of representative cultures from β-thalassemia patients, in order to identify sample characteristics compatible with palliative chemical or curative gene therapy (GT) intervention. In addition to basic vectors over-expressing β-globin, enhanced vectors (additionally down-regulating disease modifiers and aberrant β-globin mRNA species by an established shRNA co-expression strategy) have been tested in patient samples, in order to assess their potential utility as tools for personalized medicine. Results of these large-scale analyses will have wide-ranging implications for chemical and lentiviral treatments, including the establishment of markers for potentially successful chemical HbF induction and of minimum efficiency requirements for basic and enhanced GT vectors. Taken together, the expected outcome of the THALAMOSS project is a landmark shift in our approach to the treatment of β-thalassemia, based on detailed genotype-phenotype correlations, novel markers and a set of standardized analysis procedures for the stratification of patients for optimized disease management and personalized therapy.

1.5. SPECIFIC OBJECTIVES OF THE WORK PACKAGES.

WP1. The objectives of WP1 were recruitment of β-thalassaemia patients, their characterisation with respect to mutations of the β-globin genes, SNPs, HbF starting levels, phenotype, clinical pattern; the second objective of this WP was the optimization of cell cultures from peripheral blood of β-thalassemia patients. This activity provided THALAMOSS partners with consensus protocols for studies on HbF induction (WP3) and gene therapy (WP3). The activities within this WP were also connected with the supply of biological material for WP2. In addition cellular biobanks have been produced and ErPC were maintained frozen, with the possibility to the shipped to the other THALAMOSS partners as well as external collaborators.

WP2. The goal was to stratify patients with β-thalassaemic mutations at the genomic, transcriptomic and proteomic levels. Newly identified potential disease modifiers have been tested functionally. These -omics analyses provided biomarkers for therapeutic management (categorization, prognosis, diagnosis and therapy), and identify novel potential therapeutic targets. Information from this WP allowed design of DNA- and protein-based diagnostic platforms for classification of β-thalassemia patients. All data have been fed into WP4 (Data management and analysis), in order to allow correlation of genotype analyses (Task 2.1) transcriptomic data (Task 2.2) and proteomic information (Task 2.3) with clinical data (WP1) and results for therapeutic interventions (WP3) under WP4, towards the development of THALAMOSS diagnostic kits and the marker-based
prediction of treatment outcomes.

WP3. The objective of this work package was to subject ErPC cultures from patients to novel therapeutic approaches and determine their responsiveness as additional parameters in addition to the analyses performed in WP2 and subsequent patient stratification in WP4. Specifically, using standardized ErPC cultures this WP has undertaken the (a) assessment of inducibility of fetal hemoglobin (HbF) by different chemical agents; (b) evaluation of the therapeutic action of co-treatment with read-through molecules for samples with primary stop-codon mutation and (c) quantification of responsiveness to gene therapy (GT).

WP4. The major objective of this Workpackage was to build the infrastructure to enable multi-institutional gathering and processing of patient data and to define the correlation of genomics, proteomics and functional-genomics findings with medical records and cell culture analyses.

WP5. The objectives of WP5 was to facilitate collaborative exchange and technology transfer between participating laboratories, to provide high-level training and promote a dialogue with the wider scientific and lay communities across Europe on thalassemia and related societal and ethical issues. The first purpose of this WP was related to the dissemination of project progress and of the results achieved. This activity was dedicated to a wide audience, including designers, engineers and scientists from industry and SMEs, as well as researchers, teachers and students from educational institutions and clinical research centers. In addition, this activity involved Associations of the patients and their relatives. Different communication strategies have been adopted to achieve such a broad range of dissemination activities, including the set-up and maintenance of a lively web-site, user-friendly and easily-accessible. The activity regarding web-site maintenance and dissemination in a broad sense was concentrated in Tasks 5.1 and 5.2 respectively. The second goal of this WP was related to the fundamental task of project result exploitation, i.e. a committed industrial plan to turn the major outcomes of the project into real products to the market in the medium term. This plan was associated to a quantifiable socio-economical impact to the EU Society. Task 5.3 was focused on the exploitation activities, which included also market analysis and strategic road mapping. Exploitation was based on the advertisement and promotional activities for awareness creation about the outcomes of the project, as well as the preparation of the exploitation plans, both by the individual partners and by the Consortium as a whole. The means of verification for WP5 were: quality and contents of deliverables, exploitation operating procedures, time of delivery. A key objective of the exploitation task within this work package was to ensure that the regulatory framework which applies to an out-coming commercial product guarantees that its future commercial success is in no way hampered.

WP6. Ethical issues were a key concern of THALAMOSS, and this WP was proposed to ensure that they were addressed explicitly in the consortium agreement and in continuous interaction of partners within the network and with study subjects. The work package leaders had the role of providing ethical guidance to consortium members with regard to European Union regulations on research ethics. They also ensured that all research work and acquisition of samples passed the respective national research ethics committees and experimental work was carried out in compliance with national and European regulations. This WP ensured that all ethical approvals have been forwarded to the coordinating body, acquired before the start of sample utilization from thalassemia patients and included in periodic reports forwarded to the European Commission. In close collaboration with WP2 they also ensured that databases and associated patient data have been secured and inaccessible to third parties or consortium partners not directly involved in their development, maintenance and analysis, with safeguards beyond the lifetime of the proposed project.

Project Results:
DELIVERABLES OF THE THALAMOSS PROJECT.

The goals of the THALAMOSS project were expected to be achieved with the following deliverables, divided for research periods:
First period: D1.1. First List of recruited β-thalassemia patients with characterized genotype/phenotype; D1.3. Protocol A for ErPC isolation and subculturing; D1.4. Protocol B for ErPC isolation and subculturing; D1.5. Protocol C for ErPC isolation, and storage; D1.6. First β-thal cellular BioBank; D2.1. Sequencing completed of at least 50 genomic samples; D2.7. Report of cell culture standardization; D2.8. Functional analysis standardized; D2.10. Globin immunizations completed; D4.1. Analysis of requirements on data management; D4.2. THALAMOSS data management platform; D5.1. Set-up of the project web-site; D5.8. Industrial plan for the use and dissemination of the foreground, explaining how knowledge and IP issues will be managed within the consortium, between research institutions and industrial partners; D5.9. License agreements signed between the partners involved; D6.1. Report on management of biological samples from patients; D6.2. Report on management of databases on genomic DNA, RNA and proteomic profiles; D6.3. Report on management of results deriving from experiments on HbF induction; D6.4. Report on management of results based on the employment of GT technology; D6.5. Report on regulatory issues associated with cellular THALAMOSS BioBanks; D6.6. Collection of the approvals from the Bioethic Committees on the use of biological materials from thalassaemic patients; D6.7. First THALAMOSS report of gender actions; D7.6. Guidelines on information exchange of pre-existing know-how; D7.7. IPR Management Database: Pre-Existing Know-How; D7.8. IPR Management Database: First Release.


Third period: D2.13. Disease modifiers monoclonal antibodies tested in clinical samples; D3.2.2nd Report HbF Inducers. D3.4. 2nd Report on HbF Inducers with Read-through Reagents. D3.6. 2nd Report on GT Correction; D4.5. Novel whole-genome SNP analysis methods; D5.3. At least 13 publications in popular print media; D5.4. At least 24 scientific presentations at conferences/summer courses, including events organized by EU; D5.5. At least 15 publications in peer-reviewed journals; D5.6. At least two books or special volumes of scientific journals reporting the activity of THALAMOSS; D5.7. A CD-ROM outlining the THALAMOSS major achievements; D5.9. License agreements signed between the partners involved; D5.12. Market/business surveying and road mapping activity; D5.13. Guidelines for the community to be prepared for the emerging challenges in the field; D6.9. Final THALAMOSS report of gender actions; D7.3. Final Publishable Summary Report; D7.9. IPR Management Database: Final Release.

2.1. Recruitment

The total number of recruited patients thanks to the activity of partners UNIFE, CING, LGHA, UNICA, BIOCEP and CU has been more than 900. This activity was executed as planned in the first period of THALAMOSS research. In this list only patient with well characterized genotype/phenotype and actively participating to the THALAMOSS project are included. For all of them the mutations of the β-globins gene are known. The final list of genotypes of patients recruited for activities employing blood sampling, culturing of erythroid cells, isolation of genomic DNA, RNA and protein includes 127 entries contributed by UNIFE, 356 entries by CING, 22 entries by CU, 100 entries by UNICA, 5 entries by KCL and 334 entries by LGHA (total 944 entries). The analysis of the most frequent genotypes and phenotypes allows us to conclude that homozygous patients are 135
(β039/β039), 210 (β+IVSI-110/β+IVSI-110) and 21 (β+IVSI-6/β+IVSI-6). Double heterozygous patients are 58 (β039/β+IVSI-110), 95 (β+IVSI-6/β+IVSI-110) and 36 (β0IVSI-1/β+IVSI-110). In the list are present 36 homozygous sickle-cell anemia (SCA, HbS/HbS) patients. Finally, the HbS genotype was associated in 29 cases with β039, in 61 cases with β+IVSI-110, in 13 cases with β+IVSI-6 and in 17 cases with β0IVSI-1.

2.2. Biobanking

2.2.1. Introductory remarks. Biobanking of biological material, including viable cells, is a new and very relevant approach involving a wide range of public and private institutions. The number of biobanks is rapidly increasing, helping to create collaborative networks able to tackle very important biomedical issues needing of large numbers of tissue samples relating to the same groups of pathologies. In Europe, more than 600 biobanks and institutions from over 30 countries that collect samples and pathological/clinical data belong to the Biobanking and Biomolecular Resources Research Infrastructure (BBMRI-ERIC). While biobanking of biological material (cellular pellets, DNA, RNA) is important to organize collection of high-quality samples with reliable clinical information for diagnostics, therapy and research, the output is limited by the fact that experiments with biological modifiers cannot be performed. On the contrary, this is possible, at least in theory, with validated cellular biobanks, allowing a complete clinical validation.

2.2.2. Cellular biobanking is a key step in “OMICS” analyses, as well as in the screening of bioactive molecules for the development of novel therapeutic protocols. THALAMOSS has developed three culture protocols, of which one (protocol C) was used for expansion of erythroid precursors and generation of the biobank. Within the THALAMOSS project we carried out experiments finalized to (a) the generation of a cellular biobank (Thal-Biobank) from β-thalassemia patients, (b) its characterization with respect to maintenance of the phenotype (% of production of fetal hemoglobin), (c) its validation in terms of using frozen cryopreserved samples for thawing and subculturing in different laboratories for studies on induction of fetal hemoglobin (HbF) using Hydroxyurea as HbF inducer. Hydroxyurea was chosen among the different available HbF inducers, since it is already used in experimental therapy of patients affected by β-thalassemia and sickle-cell anemia.

2.2.3. The THALAMOSS centralized cellular Biobank. The cellular β-THAL Biobank is constituted of more than 1340 vials from 213 patients. The most represented genotypes are β039/β039 (102 patients, 755 cryovials), β+IVSI-6/β+IVSI-110 (35 patients, 59 cryovials), β039/β+IVSI-110 (23 patients, 160 cryovials), β+IVSI-110/β+IVSI-110 (13 patients, 120 cryovials).

2.2.4. Methods. We have demonstrated that freezing, cryopreservation and thawing steps maintain the cellular phenotype (i.e. level of HbF). In THALAMOSS protocol C, the blood is sampled in lithium-heparin (Li-He) anticoagulant and immediately used to set up cell cultures. The first step included cells stratification, using Ficoll, for separation of mononuclear cells from peripheral blood (PBMC); the second step was the selection of CD34+ cells using Miltenyi magnetic beads. Once selected, the CD34+ stem cells were placed in a 25cm2 flask containing 5ml of StemSpan medium supplemented with cytokines (CC100) and erythropoietin, then placed within a CO2 incubator at 37 °C for about five days. From the fifth day onwards after checking cells at the optical microscope for optimal growth, fresh medium is added supplemented with growth factors to keep an optimal concentration of cells of about 1x106 cells/ml. On average after 9, 11 days of expansion, cells were counted, harvested and stored in liquid nitrogen at concentration of 5x106 cells/ml/cryovial. The average number of vial collected per patient was 7±5, range 1-22 vials. The first validation of the cryopreserved cells is performed by checking the erythroid differentiation potential in terms of both kinetics and types of hemoglobin produced.

2.2.5. Validation. The validation of the cellular Thal-Biobank was consolidated by results obtained in other laboratories (CU, CHOP, UNICA, CING) on different batches of cryopreserved cells from the same patients. Also, these specimens provide an important opportunity for the research, to stratify patients based on all their phenotypic/genotypic characteristics, to evaluate the different ability of each individual to respond to inducers of HbF synthesis, and to develop novel therapeutic strategies for β-thalassemia. Correction of the biobanked cells by gene therapy and genomic editing is expected to be possible. Furthermore, generation of iPSCs is, at least in theory, a possible strategy. To our knowledge, the cellular Thal-Biobank
described (Cosenza et al., 2016, J Transl Med, 14, 255) is the only worldwide available cellular biobank for thalassemia, being a unique tool for theranostics of beta-thalassemia and (when implemented) of sickle-cell disease. An example of validation of the THALAMOSS cellular β-Thal-Biobank is shown by results demonstrating that the cryopreserved erythroid cells display biological features overlapping with those obtained by culturing in standard reference methods.

2.3. OMICS analyses

The results concerning this specific research field were several. Genomic DNA samples from more than 100 β-thalassemia patients have been used for identification of β-globin gene mutations, polymorphisms of the β-globin genes, the Xmnl polymorphism of the promoter of the fetal Gγ-globin gene, polymorphisms of the BCL11A, HBS1L-MYB and KLF1 loci (which are disease modifiers through their role in fetal γ-globin expression). DNA and RNA samples from these patients are stored for further genomic and transcriptomic analyses by the THALAMOSS consortium.

2.3.1. ASH1L: a novel marker. In a collaborative effort between EMC and KCL an English family with unexplained β-thalassemia trait was investigated which was first published in 1993 (PMID: 8101733). Through whole genome sequencing EMC/KCL have identified a region on chromosome 1 which most likely harbours the trait. Resequencing in the extended family and expression analysis of the remaining candidate genes in human erythroid progenitors revealed that a missense variant in ASH1L as the best candidate. They have performed knockdown experiments in cultured primary human erythroid progenitors to test this hypothesis. With the advent of CRISPR/Cas9-mediated genome engineering they are now introducing the missense variant in the genome to obtain definitive evidence for the causative role of ASH1L in the β-thalassemia trait phenotype.

2.3.2. The novel +25 G->A polymorphism of the Aγ-globin gene. In a study performed by UNIFE a novel polymorphism was found in β-thalassemia patients in the 5'UT sequence (+25) of the Aγ-globin gene, known to affect the LYAR (human homologue of mouse Ly-1 antibody reactive clone) binding site 5'-GGTTAT-3'. In agreement with the expectation that this mutation alters the LYAR binding activity, UNIFE found that the Aγ(+25 G->A) and γ-globin-Xmnl polymorphisms are associated with high HbF in erythroid precursor cells isolated from β039/β039 thalassemia patients. This might have an impact of THALAMOSS activity since the demonstration that the novel +25 G->A polymorphism of the Aγ-globin gene is associated in β039 thalassemia patients with high HbF in erythroid precursor cells allows to consider LYAR and LYAR-interacting proteins as novel biomarkers.

2.3.3. Phenotypic effects of HbF-associated polymorphisms. The genotyping of genetic determinants associated with increased fetal hemoglobin production (HbF) analyzed by all the partners involved in this specific activity were the rs7482144 (Xmnl) in G-gamma gene, rs11886868, rs1427407, rs10189857 in BCL11A gene, rs9399137, rs1320963, rs9402686 in HBS1L-MYB intergenic region. It was confirmed that these polymorphisms are useful for patient stratification and for prediction of the response to therapy. GWAS, transcriptomic and proteomic studies were conducted with the very interesting observation that some key feature of the β-thalassemia patients (for instance endogenous levels of HbF and/or response in vitro of ErPCs to HbF inducers) are associated with polymorphisms. For instance, the rs368698783 (+25 G ->A) polymorphism affecting LYAR binding to the Aγ-globin gene was found to be associated with high fetal hemoglobin (HbF) in β-thalassemia erythroid precursor cells treated with HbF inducers.

2.3.4. Scanning for mutations. LGHA developed a reliable, cost-effective and rapid scanning method for β-globin gene point mutations, easily adapted to a routine laboratory. The developed High-Resolution Melting Analysis (HRMA) approach is original and suitable for scanning the particularly heterogeneous β-globin gene mutations present in the Greek population, and thus adaptable to the Mediterranean and other areas where these mutations have been identified. Within this context, β-globin gene regions containing mutations frequently identified in the Greek population were divided in ten overlapping amplicons. The reactions’ setup allowed for the simultaneous amplification of multiple primer sets and partial multiplexing, thereby resulting in significant reduction of the experimental time. DNA samples from β-thalassemia patients/carriers with defined genotypes were tested. Distinct genotypes displayed distinguishable melting curves, enabling accurate detection of mutations.
The described HRMA can be adapted to a high-throughput level. It represents a rapid, simple, cost-effective, reliable, highly feasible and sensitive method for β-thalassemia gene scanning.

2.3.5. Proteomics and transcriptomics. Proteomic and transcriptomic studies allowed to identify genes differentially expressed depending on clinical parameters or HbF production; interestingly we found that transcriptomic pattern are also related to the response of HbF inducers. This observation has two theoretical and practical implications. From the theoretical point of view these results might facilitate the identification of mRNAs whose up- or down-regulation is associated with high HbF expression levels. On the other hand, our data allow to propose mRNA patterning as predictive not only of basal HbF expression, but also of response to inducer, at least in vitro. Since the response in vitro might reflect in vivo response, the mRNA patterning approach deserve to be included as molecular marker in clinical trials to determine whether is predictive of in vivo response. This issue is of great interest, since (a) it might help in decision making during the clinical managements of the β-thalassemia patients and (b) no predictive genetic and/or gene expression marker is predictive of therapeutic response in vitro. While several HbF-related polymorphisms have been described and suggested to be linked to high HbF (such as the XmnI polymorphism, and the BCL11A, MYB and KLF-1 polymorphism) their presence is not “per se” predictive of high-level production of fetal hemoglobin and in vivo response to HbF inducers. Our data on mRNA global gene expression analysis strongly suggest that full-transcriptomic might also be significant and informative with respect to a possible association with response to HbF inducers.

2.3.6. Global gene expression and HbF. Our data on mRNA global gene expression analysis strongly suggest that full-transcriptomic might also be significant and informative with respect to a possible association with response to HbF inducers. As far as global miRNA expression in ErPC cultures from β-thalassemia patients, in the experiments performed on RNA from High-HbF and Low-HbF ErPCs, 392 miRNA sequences were found expressed by all analyzed ErPC cultures. Of these sequences, 62 were found differentially expressed when High-HbF and Low-HbF samples are compared. This first set of results allows to hypothesize that this approach can discriminate between samples expressing different HbF levels.

2.3.7. Novel biochemical and molecular biology markers in β-thalassemia were identified by researchers working at THALAMOSS, among which: raptor, LYAR, KLF1, MAP3K5 gene, ASH1L, ZF-Ldb1, ZBTB7A/LRF.

These are the published evidences:

Raptor: Bianchi N et a. (PLoS One. 2015 Apr 7;10:e0121567) reported increase of microRNA-210, decrease of raptor gene expression and alteration of mammalian target of rapamycin regulated proteins following mithramycin treatment of human erythroid cells. In the first part of this study microarray analysis was performed both in mithramycin-induced K562 cells and erythroid precursors from healthy subjects or β-thalassemia patients producing low or high levels of fetal hemoglobin. It was demonstrated that: (a) microRNA-210 expression is higher in erythroid precursors from β-thalassemia patients with high production of fetal hemoglobin; (b) microRNA-210 increases as a consequence of mithramycin treatment of K562 cells and human erythroid progenitors both from healthy and β-thalassemia subjects; (c) this increase is associated with erythroid induction and elevated expression of γ-globin genes; (d) an anti-microRNA against microRNA-210 interferes with the mithramycin-induced changes of gene expression. In the second part of the study these researchers have obtained convergent evidences suggesting raptor mRNA as a putative target of microRNA-210. Indeed, microRNA-210 binding sites of its 3'-UTR region were involved in expression and are targets of microRNA-210-mediated modulation in a luciferase reporter assays. Furthermore, (i) raptor mRNA and protein are down-regulated upon mithramycin-induction both in K562 cells and erythroid progenitors from healthy and β-thalassemia subjects. In addition, (ii) administration of anti-microRNA-210 to K562 cells decreased endogenous microRNA-210 and increased raptor mRNA and protein expression. Finally, (iii) treatment of K562 cells with premicroRNA-210 led to a decrease of raptor mRNA and protein. In conclusion, microRNA-210 and raptor are involved in mithramycin-mediated erythroid differentiation of K562 cells and participate to the fine-tuning and control of γ-globin gene expression in erythroid precursor cells. The decrease of raptor gene expression was also found with other HbF inducers. As far as mechanism of action of mithramycin, Finotti et al. (Pharmacol Res. 2015 Jan;91:57-68) confirmed that...
induction of K562 cells treated with mithramycin is associated with inhibition of raptor gene transcription and mammalian target of rapamycin complex 1 (mTORC1) functions. Interestingly, the promoter sequence of the raptor gene contains several Sp1 binding sites which may explain its mechanism of action. These authors hypothesized that the G+C-selective DNA-binding drug mithramycin is able to interact with these sequences and to inhibit the binding of Sp1 to the raptor promoter due to the following results: (a) MTH strongly inhibits the interactions between Sp1 and Sp1-binding sites of the raptor promoter (studied by electrophoretic mobility shift assays, EMSA); (b) MTH strongly reduces the recruitment of Sp1 transcription factor to the raptor promoter in intact K562 cells (studied by chromatin immunoprecipitation experiments, ChIP); (c) Sp1 decoy oligonucleotides are able to specifically inhibit raptor mRNA accumulation in K562 cells. In conclusion, raptor gene expression is involved in mithramycin-mediated induction of erythroid differentiation of K562 cells and one of its mechanism of action is the inhibition of Sp1 binding to the raptor promoter.

LYAR: A polymorphism of the Aγ-globin gene is here studied in four families with β(0)-thalassemia (β(0)-IVSII-1 and β(0)-IVSI-1) and expressing unusual high HbF levels, congenital or acquired after rejection of bone marrow transplantation. This (G→A) polymorphism is present at position +25 of the Aγ-globin genes, corresponding to a 5'-UTR region of the Aγ-globin mRNA and, when present, is physically linked in chromosomes 11 of all the familiar members studied to the XmnI polymorphism and to the β(0)-thalassemia mutations. The region corresponding to the +25(G→A) polymorphism of the Aγ-globin gene belongs to a sequence recognized by DNA-binding protein complexes, including LYAR (Ly-1 antibody reactive clone), a zinc-finger transcription factor previously proposed to be involved in down-regulation of the expression of y-globin genes in erythroid cells. We found a novel polymorphism of the Aγ-globin gene in four families with β(0)-thalassemia and high levels of HbF expression. Additionally, we report evidence suggesting that the Aγ-globin gene +25(G→A) polymorphism decreases the efficiency of the interaction between this sequence and specific DNA binding protein complexes. This is a very important point and was demonstrated by electrophoretic-mobility shift assay (EMSA), docking and SPR-base biospecific interaction analysis. Docking simulations showed a marked electrostatic and shape complementarities between LYAR and the DNA consensus sequence 5'-GGTTAT-3'. Besides the expected interactions between the phosphate backbone of the nucleic acid and the largely positive surface of the protein, however, the docking simulations suggested three less obvious key interactions: (a) the ammonium ion of the LYS20 side chain interacted with the N7 of the GGTTAT, with the carbonyl of the GGTTAT and with the carbonyl of the GGTTAT; (b) the methyl functions of the GGTTAT sequence were accommodated in a cleft generated by the side chains of LYS14, ILE16 and GLN17; (c) the methyl of the thymine in the complement sequence of the GGTTAT was accommodated in a cleft generated by the side chains of MET1, LYS15 and ILE16. The results of docking simulation suggested the importance of almost all the residues in the GGTTAT sequence (or of its coupled sequence). Interestingly the G→A polymorphism does not allow the generation of one of the hydrogen bonds, suggesting that this polymorphism might be associated with decrease of LYAR-DNA recognition efficiency. Based on our SPR-based Biacore experiments and docking analysis, we can conclude that the Aγ-globin-(G→A) polymorphism, frequent in family members producing high HbF levels, modifies the binding efficiency of nuclear factors, including LYAR. This was also confirmed using purified LYAR protein. Therefore this point mutation may participate to the high HbF production exhibited by these studied patients.

KLF1: Esteghamat F, et al (2013, Blood, 121, 2553-2562) found that BCL11A downregulation in human primary adult erythroid progenitors results in elevated expression of fetal y-globin. Recent reports showed that BCL11A expression is activated by KLF1, leading to y-globin repression. To study regulation of erythropoiesis and globin expression by KLF1 and BCL11A in an in vivo model, these researchers used mice carrying a human β-globin locus transgene with combinations of Klf1 knockout, Bcl11a floxed, and EpoR(Cre) knockin alleles. They found a higher percentage of reticulocytes in adult Klf1(wt/ko) mice and a mild compensated anemia in Bcl11a(cko/cko) mice. These phenotypes were more pronounced in compound Klf1(wt/ko)::Bcl11a(cko/cko) mice. Analysis of Klf1(wt/ko), Bcl11a(cko/cko), and Klf1(wt/ko)::Bcl11a(cko/cko) mutant embryos demonstrated increased expression of mouse embryonic globins during fetal development. Expression of human y-globin remained high in Bcl11a(cko/cko) embryos during fetal development, and this was further augmented in Klf1(wt/ko)::Bcl11a(cko/cko) embryos. After birth, expression of human y-globin and mouse embryonic globins decreased in Bcl11a(cko/cko) and Klf1(wt/ko)::Bcl11a(cko/cko) mice, but the levels remained much higher than those observed in control animals. Collectively, these data support an important role for the KLF1-BCL11A axis in erythroid maturation and
developmental regulation of globin expression.

MAP3K5: Tafrali C, et al. reported in Pharmacogenomics (14, 469-483, 2013) that genomic variation in the MAP3K5 gene is associated with β-thalassemia disease severity and hydroxyurea treatment efficacy. In this study they explored the association between genetic variations in MAP3K5 and PDE7B genes, residing on chromosome 6q23, and disease severity in β-hemoglobinopathy patients, as well as the association between these variants with response to hydroxyurea (HU) treatment. Furthermore, they examined MAP3K5 expression in the context of high fetal hemoglobin (HbF) and upon HU treatment in erythroid progenitor cells from healthy and KLF1 haploinsufficient individuals. A short tandem repeat in the MAP3K5 promoter and two intronic MAP3K5 gene variants, as well as a PDE7B variant, are associated with low HbF levels and a severe disease phenotype. Moreover, MAP3K5 mRNA expression levels are altered in the context of high HbF and are affected by the presence of HU. Lastly, the abovementioned MAP3K5 variants are associated with HU treatment efficacy. These data suggest that these MAP3K5 variants are indicative of β-thalassemia disease severity and response to HU treatment.

ASH1L: ASH1L was proposed (these results have been already discussed 2.3.1) as involved in globin gene regulation through functional mRNA knock-down and chromatin-immunoprecipitation studies in human erythroid progenitor cells.

ZF-Ldb1: This research activity started with the very interesting observation publish by Deng et a. (Cell 158:849-60, 2014) demonstrating that reactivation of developmentally silenced globin genes was possible by forced chromatin looping, a common features enabling distal enhancers to contact target promoters. Interestingly, in erythroid cells, the locus control region (LCR) contacts β-type globin genes in a developmental stage-specific manner to stimulate transcription. Previously, we induced LCR-promoter looping by tethering the self-association domain (SA) of Ldb1 to the β-globin promoter via artificial zinc fingers. Here, we show that targeting the SA to a developmentally silenced embryonic globin gene in adult murine erythroblasts triggers its transcriptional reactivation. This activity depends on the LCR, consistent with an LCR-promoter looping mechanism. Strikingly, targeting the SA to the fetal γ-globin promoter in primary adult human erythroblasts increases γ-globin promoter-LCR contacts, stimulating transcription to approximately 85% of total β-globin synthesis, with a reciprocal reduction in adult β-globin expression. Our findings demonstrate that forced chromatin looping can override a stringent developmental gene expression program and suggest a novel approach to control the balance of globin gene transcription for therapeutic applications. In a second study, Breda et al. (Blood. 2016 Aug 25;128(8):1139-43) were able to demonstrate that Forced chromatin looping raises fetal hemoglobin in adult sickle cells to higher levels than pharmacologic inducers. In adult erythroid cells, the LCR can be redirected from the adult β- to the fetal γ-globin promoter by tethering Ldb1 to the human γ-globin promoter with custom-designed zinc finger (ZF) proteins (ZF-Ldb1), leading to reactivation of γ-globin gene expression. To compare this approach to pharmacologic reactivation of fetal hemoglobin (HbF), hematopoietic cells from patients with SCD were treated with a lentivirus expressing the ZF-Ldb1 or with chemical HbF inducers. The HbF increase in cells treated with ZF-Ldb1 was more than double that observed with decitabine and pomalidomide; butyrate had an intermediate effect whereas tranylcypromine and hydroxyurea showed relatively low HbF reactivation. ZF-Ldb1 showed comparatively little toxicity, and reduced sickle hemoglobin (HbS) synthesis as well as sickling of SCD erythroid cells under hypoxic conditions. The efficacy and low cytotoxicity of lentiviral-mediated ZF-Ldb1 gene transfer compared with the drug regimens support its therapeutic potential for the treatment of SCD.

ZBTB7/LRF: This novel repressor of the γ-globin gene transcription was studied by Norton et al. (Blood Advances, 2017,1, 685-692). As pointed out several times within THALAMOSS reports and deliverables, the γ-globin repressor BCL11A has become the major focus, with several studies investigating its regulation and function as a first step to inhibiting its expression or activity. However, a second repression mechanism was recently shown to be mediated by the transcription factor ZBTB7A/LRF, suggesting that understanding the regulation of ZBTB7A may also be useful. The authors show that Kruppel-like factor 1 (KLF1) directly drives expression of ZBTB7A in erythroid cells by binding to its proximal promoter. They have also uncovered an erythroid-specific regulation mechanism, leading to the upregulation of a novel ZBTB7A transcript in the erythroid compartment. The demonstration that ZBTB7A, like BCL11A, is a KLF1 target gene also fits with the observation that reduced KLF1 expression or activity is associated with HbF repression.
2.4. Novel diagnostic tools: monoclonal antibodies

The first set of results obtained allow to conclude that a number of heavy chain only antibodies have been isolated using peptide immunizations in the proprietary Harbour Antibodies mice. This resulted in the isolation of a number of HCAb specific for the β-globin chain and some candidate antibodies that also recognize sickle cell beta globin. The data obtained show that the isolated β-globin specific HCAb are functional in clinical samples. After comparison with a commercially available anti-β-globin antibody, we concluded that antibodies developed at Harbour are good candidates for further development of β-globin assays. A number of heavy chain only antibodies against other disease modifiers have been isolated using peptide immunizations in the proprietary Harbour Antibodies mice. Antibodies against erythroid markers and disease modifiers were characterized in clinical setting using different cellular and biochemical approaches, such as FACS analyses, Surface Plasmon Resonance (SPR) biosensor-base analyses, Western blotting procedures.

2.5. Novel experimental systems developed at THALAMOSS

The development of in vitro and the characterization of in vivo model systems was a key issue in THALAMOSS.

2.5.1. The HUDEP2 cell line. One interesting developed and characterized system was constituted by HUDEP2 cells, suitable for studies on erythroid differentiation and γ-globin reactivation. EMC performed lentiviral shRNA mediated knockdown of known γ-globin suppressors. EMC observed that knockdown of fetal globin suppressors induced γ-globin expression to high levels, while γ-globin remained low in the HUDEP2 cells that were treated with control shRNA constructs, confirming that HUDEP2 cells are a valuable addition to the arsenal of experimental systems available.

2.5.2. Development and characterization of K562 cell clones expressing at high levels BCL11A. This research activity was conducted by UNIFE and IRBM. Twelve K562 clones expressing different levels of BCL11A-XL were characterized and found that a clear relationship does exist between the levels of BCL11A-XL and the extent of differentiation. Using mithramycin as inducer, we found that MTH was unable to induce differentiation in K562 cell clones expressing high levels of BCL11A-XL. By sharp contrast MTH was able to induce the increase of γ-globin mRNA in K562 clones expressing intermediate levels of BCL11A-XL, suggesting that BCL11A-XL activity is counteracted by MTH. These clones might be proposed to identify novel HbF inducers acting inhibiting BCL-11A.

2.5.3. Humanized murine erythroleukemia (MEL) cells. In order to facilitate the reproducible evaluation of HBBIVSI-110(G>A)-specific therapeutic approaches CING created a robust heterologous expression system, based on murine erythroleukemia (MEL) cells. These MEL-HBBIVSI-110(G>A) cells, while lacking HBBIVSI-110(G>A)-related disease pathology and human-specific disease modifiers, would have several advantages for initial screening of HBBIVSI-110(G>A)-specific therapies. To wit, the transgenic MEL cultures would be easy to maintain and scale up, would allow erythroid differentiation and high-level expression from control elements of the human β-globin locus, and nucleotide sequences and proteins stemming from the human transgene would be easily distinguishable from and thus quantifiable against the murine background. To produce the envisaged model CING first introduced the HBBIVSI-110(G>A) mutation into the GLOBE transfer vector plasmid. The corresponding GLOBEIVSI-110(G>A) LV and the original GLOBE LV were then used to transduce MEL cells and produce humanized cell lines MEL-HBBIVSI-110(G>A) and MEL-HBB with average VCNs of 1.9 and 2.0 respectively, in bulk populations. We additionally applied two rounds of limiting dilutions to isolate a clonal MEL-HBBIVSI-110(G>A) cell line with VCN 1. Immunoblots of MEL-HBBIVSI-110(G>A) and MEL-HBB cell pools and of the MEL-HBBIVSI-110(G>A) VCN 1 clonal cell line for three independent differentiation experiments revealed that both mutant cell lines express HBB at below 7% of the positive control (Fig 3.2). These findings correspond to HBB expression levels observed in HBBIVSI-110(G>A) mice and in HBBIVSI-110(G>A)-homozygous patients relative to controls. We thus concluded that the heterologous cell model would be informative for the evaluation of mutation-specific therapeutic approaches for HBBIVSI-110(G>A) thalassemia. In the following, MEL-
HBBIVSI-110(G>A) VCN 1 was used to analyze the effect of HBBIVSI-110(G>A)-specific RNAi on HBB expression in transgenic MEL cells.

2.5.4. Dual-fluorescence reporter in yeast. In order to develop novel screening systems for the identification of novel read-through reagents, Altamura et al. presented a rapid, sensitive and quantitative method based on a dual-fluorescence reporter expressed in the yeast Saccharomyces cerevisiae to monitor and quantitate read-through at PTCs (PLoS One. 2016 Apr 27; 11(4): e0154260). They have shown that the novel system works equally well in detecting read-through at all three PTCs UGA, UAG and UAA.

2.6. Functional studies

During the THALAMOSS OMICS analyses the global expression of miRNAs were also analyzed. We found a clear indication of a microRNA pattern associated with high-HbF production by ErPCs isolated from β-thalassemia patients. MicroRNAs (miRNAs or miRs) are a family of small (19 to 25 nucleotides in length) non-coding RNAs that regulate gene expression by sequence-selective targeting of mRNAs, leading to translational repression or mRNA degradation, depending on the degree of complementarity with target mRNA sequences. Since their discovery and first characterization, the number of miRNA sequences deposited in the miRBase databases is increasing. Considering that a single miRNA can target several mRNAs and a single mRNA may contain several signals for miRNA recognition in the 3’UTR sequence, it has been calculated that at least 10–40% of human mRNAs are a target for miRNAs. This specific field of miRNA research has confirmed that the complex networks constituted by miRNAs and mRNA targets coding for structural and regulatory proteins lead to the control of highly regulated biological functions, such as differentiation, cell cycle and apoptosis. The low expression of a given miRNA is expected to be linked with a potential expression of target mRNAs. Conversely, the high expression of miRNAs is expected to negatively affect the biological functions of target mRNAs. Alterations in miRNA expression have been demonstrated to be associated with a variety of human pathologies (including thalassemia) and the guided alterations of specific miRNAs have been suggested as novel approaches for the development of innovative therapeutic protocols. miRNA therapeutics is a novel field in which miRNA activity is the major target of intervention. The inhibition of miRNA activity can be readily achieved by the use of small miRNA inhibitors, oligomers, including RNA, DNA and DNA analogues (miRNA antisense therapy). On the contrary, an increase in miRNA function (miRNA replacement therapy) can be achieved by the use of modified miRNA mimetics, such as plasmid or lentiviral vectors carrying miRNA sequences. This was made possible through a growing understanding of the regulation of globin switching, also by regulatory microRNAs (miRNAs), and of transcriptional repressors of γ-globin as therapeutic targets. Within THALAMOSS, the activity of miR-96 and miR-210 were analyzed demonstrating that they play a role in HbF expression levels and are modulated during HbF induction of in vitro culture ErPCs from β-thalassemia patients.

As far as transcription factors, among the candidate target genes for knockdown is the zinc finger transcription factor Krüppel-like factor 1 (KLF1, also known as the erythroid Krüppel-like factor, EKLF), which acts as an erythroid-specific master switch of globin gene expression and whose autonomy in directing globin gene expression is underlined by the observation that the mere insertion of a KLF1 binding site into the human δ-globin promoter confers developmental inducibility and a reduction of the thalassemia phenotype in mice. Besides KLF1, Oct-1, MYB, and BCL11A have been identified as repressors of γ-globin gene transcription. For instance, the zinc finger transcription factor BCL11A has recently been shown to function as a repressor of HbF expression, with transgenic deactivation of BCL11A reactivating HbF and correcting a humanized sickle Hb mouse model and with BCL11A knockdown leading to significant HbF induction in human cells, similar to knockdown of its positive regulator KLF1. Moreover, compound Klf1::Bcl11a mutant mice that carry the human β-globin locus showed further enhanced γ-globin expression compared to single-mutant animals, indicating that a strategy targeting both genes together (without affecting non-erythroid functions of BCL11A) might have additional therapeutic benefits in β-thalassemia. In order to move transgene-mediated activation of γ-globin from concept to therapeutic application, shRNA expression from constitutive RNA polymerase III promoters, such as the commonly used U6 promoter, needs to be avoided. To this end, a BCL11A-specific shRNA with the flanking sequences of a naturally occurring miRNA (miR223), allowing its (potentially regulated) expression from RNA
polymerase II-driven promoters. Using lentiviral vectors for spleen focus-forming virus (SFFV)-promoter-driven BCL11A shRNAmiR expression in murine erythroleukemia cells, approximately 50% of control embryonic γ levels were achieved compared to the equivalent positive U6 shRNA control, so that controlled and stable shRNA-mediated HbF induction has achieved an efficiency of potential clinical relevance. Novel transcription factor or transcription factors complexes identified and/or characterized under THALAMOSS have been LYAR, ZF-Ldb1, ZBTB7/LRF.

2.7. Novel therapeutic approaches: fetal hemoglobin induction and correction of stop codon mutations

2.7.1. Introductory remarks. Treatment of β-thalassemia is symptomatic and it is generally accepted that it can still be considered a major unmet medical need. Therefore, β-thalassemias belongs to the class of diseases without an adequate treatment. Survival is increased, even in patients needing transfusions, in comparison with a few years ago in most countries, but the quality of life is still poor for many patients and the complication(s) of frequent transfusions is/are a major problem. In some β-thalassemia patients, an anomalous expression of gamma-globin genes has been observed, with a consequent rise in HbF (Fetal Hemoglobin) levels from 2.5% to 20% or more. The cases with very high HbF levels display a clinical phenotype known as HPFH (Hereditary Persistence of Fetal Hemoglobin) and exhibit a positive clinical status, since the activation of gamma-globin genes is associated with HbF increase, and this partly overcomes the problems caused by the lack of HbA (Adult Hemoglobin) in thalassemia syndromes.

2.7.2. Novel HbF inducers and characterization of known HbF inducers. With the aim of mimicking HPFH phenotypes, several compounds have been evaluated able to induce expression of embryo-fetal hemoglobins. In particular, Hydroxyurea (HU) is frequently used with mixed results, despite the fact that only a limited fraction of patients respond properly and some of them become resistant to treatment. In addition, beta-thalassemia patients might become non responders to HU after long-term treatment. Therefore, new HbF inducers are highly needed.

The list of the class of HbF inducers so far analyzed is the following:

1. Psoralen and angelicin derivatives
2. Mithramycin, including its mechanism of action;
3. Rapamycin, including its mechanism of action;
5. Novel HDAC inhibitors identified by high throughput screening.
6. Novel isoxazole analogues exhibiting very high levels of HbF induction.

2.7.3. Butyric acid analogues. We have identified the basal chemical structures of new inducers of fetal hemoglobin starting from the structure of butyric acid. In this respect, we have considered a variety of butyric acid analogues and metabolites, starting from those reported in the U.S. Patent number 5,700,640. The structures of these compounds are stringently required for erythroid differentiation ability: for example, the fatty acid chains longer than the 4-carbon butyric acid did not show activity. We have identified one compound exhibiting very high HbF inducing activity. The results from the first screening performed on K562 cells show that the one compound (compound 4174), containing a cyclopropane ring obtained by cyclization of butyric acid, exhibits erythroid differentiation activity without anti-proliferative effects. The compound 4174 demonstrated HbF induction activity on ErPCs.

2.7.4. Mechanism of action of mithramycin. We have reported studies on the mechanism of action of mithramycin, sustaining the possible inhibitory effects on the molecular interactions between the Sp1 transcription factor and the promoters of the raptor and BCL11A genes. One of the mechanism of action of mithramycin is a repression of the transcription of the BCL11A gene, and the second is the inhibition of the binding of the BCL11A complex to the γ-globin gene promoter. In addition mithramycin could also act through up-regulation of the microRNA miR-210, thereby down-regulating the miR-210 targets, including raptor.
2.7.5. Sirolimus (rapamycin). Within this framework, Sirolimus (rapamycin) is particularly interesting as an inducer of fetal hemoglobin, in particular since it has been used for many years for different indications and thus has a well-established safety profile. As far as possible HbF inducers of therapeutic interest studies on rapamycin (Sirolimus) were included in the present deliverable. The results indicated that: (a) Sirolimus increases HbF in cultures with different basal HbF levels; (b) Sirolimus increases the overall Hb content/cell; (c) Sirolimus selectively induced γ-globin mRNA accumulation, with only a minor effect on β-globin and no effect on α-globin mRNAs; (d) there is a strong correlation between the increase by Sirolimus of HbF and the increase in γ-globin mRNA content. UNIFE started a collaborative effort with RARE-PARTNERS to obtained the designation of ORPHAN DRUG for rapamycin. The possible use of rapamycin in clinical trials is sustained by the complete analysis performed under the last year of the THALAMOSS project on the effects of this mTOR inhibitor on 54 ErPC cultures obtained from 38 β-thalassemia and SCD patients.

2.7.6. From laboratory to the clinical trial: Sirolimus. This open a novel options for the treatment of β-thalassemia and sickle-cell anemia patients. The ODD (Drug Designation) status was granted by EMA (European Medicines Agency) on 12 November 2015 to Rare Partners (RP, Italy), (OD/142/15), the indication being “Treatment of beta thalassaemia intermedia and major”. This followed the positive opinion issued by the Committee for Orphan Medicinal Products (COMP). ODD status was also granted by U.S. Food and Drug Administration (FDA) to Sirolimus for the treatment of beta thalassaemia. The decision has been communicated by the Agency on June 21 2016. Recommendation was provided for the designation of the medicinal product containing Sirolimus as an orphan medicinal product for the indication: treatment of beta thalassaemia intermedia and major. A request for Protocol Assistance submitted to EMA in July/August 2016 and evaluated in September 2016. The proponents have participated to a meeting at EMA on September 26, 2016 discussing the Sirolimus clinical trial for beta-thalassemia (EMA/CHMP/SAWP/597334/2016). We have described a plan for the initial phase of clinical development and applied for Protocol Assistance to EMA.

2.7.7. Resveratrol and resveratrol analogues. We have also reported studies on resveratrol and analogues as possible HbF inducers. Since increasing concentrations of resveratrol resulted in a dose dependent reduction in cell survival, derivatives were studied which exhibit potent HbF-inducing properties with lower cytotoxicity. Out of the nine derivatives tested in K562 cells, three exhibited hemoglobin inducing activity comparable to the parent compound, one of which being less cytotoxic. Novel HbF inducers were characterized and recently patented molecules obtained by IRBM were further characterized and validated. Finally, comparisons between HbF inducers were conducted.

2.7.8. Comparison of different HbF inducers. In a THALAMOSS study four agents were selected: (1) Lenalidomide, member of a class of immunomodulators used as anticancer agents, (2) Angelicin, a DNA binding compound structurally related to psoralens, (3) 5-aza-2'-deoxycytidine (decitabine), an antimetabolite nucleoside analogue and (4) Mithramycin, another DNA-binding agent.

2.7.9. Isoxazole analogues. The data obtained from RT-PCR and HPLC analyses demonstrate that these isoxazoles compounds are very active in inducing HbF, even in ErPCs not induced by HU. These effects are concentration dependent. This part of THALAMOSS research is under IP and a patent was written and is under submission.

2.7.10. Response to HbF inducers and genetic and phenotypic parameters. Most of the data obtained clearly demonstrate that the XmnI polymorphisms is one of the polymorphisms (together with BCL11A and MYB polymorphisms) that can be used for the recruitment of β-thalassemia patients who retain the highest probability of Sirolimus-mediated HbF induction. Another interesting parameter is related to the HbF staring levels. The data obtained support the concept that we are able to screen for patients expected to respond to Sirolimus treatment. In fact a good relationship does exist between response to sirolimus treatment and endogenous levels of HbF (which is known in most patients).

2.7.11. Reduction of α-aggregates. One of the important effect of an HbF inducer is the induced decrease of α-aggregates,
which is clinically relevant. The unbalanced content of α-globin and β-globin chain is in fact one of the biochemical basis for the physiopathological features of β-thalassemia. An example was recently reported for isoxazoles, which were found to be much more active than HU in decreasing α-aggregates in treated ErPC cultures.

2.7.12. Personalized response of ErPCs to isoxazoles. The studies performed using all the isoxazole on ErPCs from β-thalassemia patients demonstrate that isoxazoles are more active than HU in inducing HbF in ErPCs responsive to HU. Moreover, and most important, some isoxazoles are able to induce HbF in ErPCs resistant to HU mediated HbF induction. It should be concluded that the effects are to some extent heterogenous within the studied β-thalassemia ErPC population. This is expected, since it is well known that each patient might differentially respond to HbF inducers.

2.7.13. Targeting BCL11A. Induction of HbF can be achieved following targeting of the γ-globin gene repressor BCL11A. We have demonstrated increase of γ-globin gene expression following (a) targeting of BCL11A with decoy molecules mimicking the DNA sequences bound by the BCL11A complex; in addition (b) we inhibited BCL11A by cell transfection with pre-miR210 (able to target BCL11A mRNA) and antisense PNAs. Demonstrating the utility of the RP-HPLC method developed under THALAMOSS, our study established several to the best of our knowledge novel findings for the gene therapy of hemoglobinopathies. First, BCL11A knockout preferentially induces the fetally predominant Gγ-globin expression and to a lesser extent the Aγ-globin gene, which represents the predominant γ-globin in adult blood. Second, at least for BCL11A and the designer nucleases tested, targeting the start codon for NHEJ-mediated disruption is no more efficient for the creation of functional knockouts than targeting a proximal region of exon 1. Third, we demonstrate the constraints on protein expression and competition at the β-globin locus for in vitro differentiation of gene-edited primary human HSCs of a β-thalassemia carrier, by showing that, as for carriers in vivo, induction of γ-globin expression does not alter the overall balance of α- to β-globin chains but instead leads to concomitant down-regulation of β-globin.

As far the possible use of transfection with pre-miRNAs and BCL11A expression miR-210 was studied. The first results was the identification within the BCL11A mRNA of a possible target site for miR-210. The following results sustain this hypothesis: (a) interactions between miR-210 and the miR-210 BCL11A site were demonstrated by SPR-based biomolecular interaction analysis (BIA); (b) the miR-210 site of BCL11A-XL is conserved through molecular evolution; (c) forced expression of miR-210 leads to decrease of BCL11A mRNA and increase of γ-globin mRNA content in erythroid cells, including erythroid precursors isolated from β-thalassemia patients. Our study suggests that the mRNA sequence of BCL11A can be targeted by miR-210. In addition to the theoretical point of view, these data are of interest from the applied point of view, supporting a novel strategy to inhibit BCL11A by mimicking miR-210 functions, accordingly with the concept supported by several papers and patent applications that inhibition of BCL11A is a recognized strategy for fetal hemoglobin (HbF) induction for treatment of β-thalassemia.

2.8. Inhibiting the α-globin gene expression.

In addition to HbF inducers, we attempted to inhibit globin gene expression. This might be important, since clinical complications in β-thalassemia and other hemoglobinopathies (such as sickle-cell anemia, SCA) are related also to production of defective proteins (the β-globin in SCA) or to accumulation of free globins which are not organized in a functional tetramer (such in the case of free α-globins in β-thalassemia). In a first proof-of-concept paper peptide nucleic acids (PNAs) were employed to alter globin gene expression. The main conclusion of the the results obtained was that PNAs designed to target adult murine β-globin mRNA inhibit hemoglobin accumulation and erythroid differentiation of murine erythroleukemia (MEL) cells with high efficiency and fair selectivity. No major effects were observed on cell proliferation. Our study supports the concept that PNAs may be used to target mRNAs that, similar to globin mRNAs, are expressed at very high levels in differentiating erythroid cells. Our data suggest that PNAs inhibit the excess production of globins involved in the pathophysiology of hemoglobinopathies. This approach can be applied to inhibition of expression of α-globin genes. The reduction of the excess of free α-globin chain was also discussed in D3.2 where we reported data suggesting a close link. 

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between efficiency of HbF induction and parallel reduction of free α-globins.

2.9. Novel therapeutic approaches: gene therapy

Over the last two decades, major efforts have been made to achieve therapeutic levels of exogenous β-like globin chains in β-thalassemia and SCA. These finally came to fruition when a switch from γ-retroviral vectors to lentiviral vectors allowed the efficient transduction of non-dividing cells with a sufficiently large expression cassette, encouraging numerous research groups to work towards vectors expressing β-globin, anti-sickling variants of β-globin and γ-globin.

2.9.1. CU/CHOP and UNIFE have used the AnkT9W vector, generated by modifying a previously characterized vector (TNS9), with the aim of increasing its safety and efficiency. For this purpose the 3’ long terminal repeat (3’ LTR) was disarmed by deleting the U3 region (self inactivating-LTR or SIN-LTR). The deletion, which includes the TATA box and all the major determinants responsible for regulating the HIV-1 promoter, abolished the LTR promoter activity, but did not affect vector titers or transgene expression in vitro. The cis-acting woodchuck post regulatory regulatory element (WPRE) was also introduced, to increase viral production and titer. In addition, in the 3’ SIN-LTR was introduced an erythroid-specific ankyrin 5’ hyper-sensitive (HS) barrier insulator. AnkT9W is less prone to silencing in vitro and achieves remarkably high levels of Hb synthesis in thalassemic mice than previous characterized vector, such as TNS9. The data obtained by CU/CHOP indicate that the ankyrin insulator acts like a barrier element, increasing gene expression and reducing silencing at randomly integrated chromosomal locations.

2.9.2. Furthermore, THALAMOSS correlated the improved ability of novel vectors to correct the thalassaemic phenotype with its enhanced ability to transcribe the transgenic β-globin mRNA during the early phases of erythropoiesis and, as a consequence, translation. CING studied the GLOBE base vector and a modified series of vectors based on the GLOBE vector to pertaining subgroups of patient samples. These vectors co-express with β-globin (a) shRNAs against negative regulators of foetal haemoglobin, aiming to enhance expression of the endogenous γ-globin gene and (b) shRNAs specific for aberrant thalassaemic mRNAs aiming to remove or deplete potentially interfering non-functional mRNAs. These vectors are currently under study and will help highlight different sub-groups of patients who would benefit from customised GT vectors, as identifiable through the routine stratification techniques this project will develop under WP4. In line with technical developments in the field and the emergence of easily accessible high-efficiency gene editing, this strategy has been modified. The strategy of intronically expressed shRNAs required substantial optimization, also in the hands of others, and has been replaced as a mutation-specific approach by genome editing applications which are only now being transferred to analyses in ErPCs.

The overall conclusion are the following: an ankyrin element can increase β-globin expression in lentiviral vectors. Using this element, a lentiviral vector was generated named AnkT9W, which contains the same β-globin sequences as TNS9, with the addition of the ankyrin element in the 3’ self-inactivating (SIN) LTR. The ankyrin element increased RNA expression during each stage of erythroid differentiation due to an increased binding of PolII to the β-globin promoter and overall synthesis of the β-globin protein. Furthermore, the presence of the ankyrin element improved HbA production compared to the parental TNS9 vector in MEL cells and mice affected by β-thalassemia intermedia, Hbbth3+. In a further research effort CHOP generated novel vectors modifying the sequence of the β-globin gene included in AnkT9W to improve its regulation. Some of them were further modified to implement the LCR regulatory region. Additional changes included repositioning of the Woodchuck Post-Regulatory Element (WPRE) outside the integrating portion of the lentiviral genome to preserve its ability to increase viral titer, and inclusion of a strong bovine growth hormone polyA signal, which has been shown to increased lentiviral titers and transgene expression. Regardless of all these changes, our data indicate that our new constructs maintain robust viral titers.

2.10. The THALAMOSS Cohort data set

The development and deployment of the THALAMOSS Data Management Platform was based on specific requirements of the
clinicians and results of biochemical analyses as well as production of OMICS data. The THALAMOSS Data Management Platform includes finer-grained representation of genotyping, enhanced and restructured clinical parameters, support for raw and processed omics data with particular attention to genomics and proteomics, as well as new parameters representing results of various types of laboratory analyses. All the analytical results support also information about the specific analytical methods used for generating the data, in order to ensure reproducibility of the results, to support validation of measurements, and to support semantically correct harmonization of results. At the end of the project, the cohort in the THALAMOSS Data Management Platform included 663 patients and one cell line, 42 RNA-seq analyses and 9x6 proteomic analyses, and sequencing data coming from cell lines (overall size of omics data totals to 204GB). In order to ensure findability, the THALAMOSS Cohort is advertised in the BBMRI-ERIC Directory. Because the cohort includes pseudonymized data, the accessibility is controlled by the data access committee, where each of the THALAMOSS partners contributing to the data set is represented; this approach will ensure that all the legal and ethical considerations are taken into account when assessing access requests, including those imposed by the national/local regulations on the contributing institutions.

2.10.1. Bioinformatics analyses and patient stratification with biomarkers. Based on experiences with the complex data model for the THALAMOSS Data Management Platform, a general specialized software tool was designed and developed that would traverse the database schema for any given combination of variables and formatting requirements to i) generate the necessary SQL command and ii) carry out data selection and formatting, writing the results in denormalized (flat-file) format, suitable for statistical analysis, machine learning or visualization. We designed an algorithm involving efficient graph traversal and SQL JOIN statement composition which was subsequently implemented in Python and rigorously tested. This work was presented at the 2015 IWBBIO conference in Granada and published as a peer-reviewed paper in a LNSC proceedings volume (Stefanic and Lexa, 2015). As a proof-of-principle, we have performed a clustering-based and machine-learning-based stratification techniques applied to selected clinical data and marker subsets from thalassemia patients (Vitamin D, T2 Liver, T Score Spine, T Score Femur, Ferritin, Diabetes etc). We have performed a computational analysis with 102 thalassemia patients collected from Limassol Hospital (Cyprus). The range of the age of the patients was between 3-68 years and the following variables as possible inputs to the model were available: Vitamin D, T2 Liver, T Score Spine, T Score Femur, Ferritin and Diabetes. After the initial analysis and due to the lack of some data we proceed with 97 patients. The specific patients were clustered in two categories (61 patients with low vitamin D3 and 36 patients with vitamin D3 >30). The available data was split into training and test set (68 Vs 29 patients respectively). The attribute selection was based on training data and was done using as an evaluator the InfoGainAttributeEval and Ranker as the search method. For the modelling methodology we have used as a classifier kNN (k-Nearest Neighbors algorithm). The produced models have been subsequently tested for accurate class predictions with an independent set of samples. For this goal we have built KNIME workflows (analyzing data and automatically reporting) and have developed custom-made KNIME nodes (called Enalos KNIME nodes http://www.novamechanics.com/knime.php) drawing on the user friendly and comprehensive open-source data integration. In terms of marker discovery, the availability of the denormalization techniques mentioned above made it relatively easy to carry out several types of all-versus-all analyses on the collected data. As a first approximation of such analysis we are creating correlation and regression networks for all the studied variables.

2.10.2. Biomarker and genotype-phenotype public database. Characteristics of the infrastructure. A. Server Software. The server is a Linux server with an installed Apache Tomcat as web server. Apache Tomcat is capable of running JSP pages as well servlets and in general has JAVA capabilities which we used to create the web service. For the development of the models we plan to use KNIME analytics platform and our in house development Enalos and Enalos+ KNIME nodes. The server has the ability to export as a web service, with a GUI, every workflow developed in KNIME and extract data from every database or other web services with API. As possible inputs, any data that can be read by KNIME (i.e. CSV file, XLS, txt files etc.) can be used and then we can export any model developed in KNIME as a web service. The server has the ability to store multiple workflows (models) that can be accessed online. Among others, the web service offers automatic reporting. B. Architecture. The web service is designed in a way that makes it easy for the administrator to add as many new models as s/he wishes without knowing the architecture and the design of the web service. There are three tiers for the web service. The first tier is the interface between the user and the web service where the user can insert the required input data in two ways to initiate a
prediction: (1) the user inserts the values that are necessary for the workflow in html input boxes which are designed for a specific workflow and (2) the user uploads a .csv file that contains the same headers as those shown in the manual form of insertion of data and it is preferable when large amount of data is available. In the second tier a simple system to call and execute the workflows is implemented. There is no limit to the number of the results files but only a restriction that are being written in a specific result folder. Finally, in the third tier the result files are made available to the user. Our web service goes to this result folder and these files are presented as links to the user and s/he can download them to her/his PC by clicking them if they are of CSV or PDF format or see the results if they are in a HTML format.

2.11. Diagnostics: novel approaches for personalized therapy and non invasive prenatal diagnosis

2.11.1. Introductory remarks. Biological tests and genetic analyses for diagnosis and characterization of hematological diseases in health laboratories should be designed and developed with the aim of meeting the major medical needs of hospitals and pharmaceutical companies involved in this field of applied biomedicine. Basically, biological tests involve adult/newborn subjects, while genetic analyses involve adult thalassemia patients, newborns, embryos/fetuses, pre-implantation embryos and pre-fertilization oocytes. Genetic testing approaches to perform diagnosis consist of molecular techniques, that should be absolutely reproducible, fast, sensitive, cheap, portable. For instance, polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) are the two common techniques employed on a single or two cells obtained via embryo biopsy during pre-implantation diagnosis. They are useful mainly for diagnosis and/or prognosis, even though recent studies point out the need of genetic information for the development of personalized therapy.

Since the discovery of cell-free fetal DNA (cffDNA) in maternal plasma, diagnostic non-invasive prenatal methods have been developed or optimized for fetal sex determination and identification of genetic diseases. As far as fetal sex determination, this might be important for therapeutic intervention on sex-associated pathologies such as Duchenne muscular dystrophy, hemophilia and congenital adrenal hyperplasia. Surface plasmon resonance (SPR)-based biosensors might be useful for these studies, because they allow to monitor the molecular interactions in real-time providing qualitative and quantitative information, through kinetics, affinity and concentration analyses. Within THALAMOSS the activity on molecular diagnosis was focused on three issues: (a) demonstrate the feasibility of SPR-based biosensors for real-time detection of Y-sequences; (b) develop pre-natal diagnostic approaches for β-thalassemia; (c) verify whether SPR-based prenatal diagnosis can be applied to β-thalassemia.

2.11.2. SPR-based non-invasive prenatal diagnosis: detection of Y-chromosome sequences. In a first study the Biacore™ X100 has been applied to identify Y-chromosome sequence in cffDNA obtained from plasma samples of 26 pregnant women at different gestational ages. We have performed SPR-based analysis of SRY PCR products using SRY-specific probes immobilized on the sensor chip. We have demonstrated that there is a statistically significant difference between samples collected by pregnancies carrying male or female fetuses. Moreover, cffDNA obtained at early gestational ages and not detectable by conventional quantitative real-time PCR can be discriminated with high accuracy and reliability using SPR-based biosensors.

2.11.3. Novel genotyping assays. In a second study Four TaqMan® genotyping assays for the most common β-thalassemia mutations present in the Mediterranean area were designed and validated for the genotype characterization of genomic DNA extracted from 94 subjects comprising 25 healthy donors, 33 healthy carriers and 36 β-thalassemia patients. In addition, 15 specimens at late gestation (21-39 gestational weeks) and 11 at early gestation (5-18 gestational weeks) were collected from pregnant women, and circulating cell-free fetal DNAs were extracted and analyzed with these four genotyping assays. We developed four simple, inexpensive and versatile genotyping assays for the postnatal and prenatal identification of the thalassemia mutations β039, β+IVSI-110, β+IVSI-6, β0IVSI-1. These genotyping assays are able to detect paternally inherited point mutations in the fetus and could be efficiently employed for non-invasive prenatal diagnosis of β-globin gene mutations, starting from the 9th gestational week.
2.11.4. Prenatal non-invasive diagnosis of thalassemia: a proof of principle. In the third study we have focused on the possible application of the developed SPR-based techniques as a non-invasive diagnostic tool to identify genetic point mutations such as the β+ IVSI-110 responsible of a β+ thalassemia phenotype. This non-invasive diagnostic strategy for the detection of the β+ IVSI-110 thalassemia mutation inherited from the father, was applied to blood samples obtained from pregnant women at different gestational ages. In order to confirm the father mutation or analyze the fetus genotype for the β+ IVSI-110 thalassemia mutation, salivary swabs were collected, and genomic DNAs were extracted and sequenced. Three plasma samples from pregnant women were available with father carriers of β+ IVSI-110 thalassemia mutation, collected at the 37th, 15th and 37th gestational week, respectively. In order to obtain a detectable amount of PCR product for the hybridization with the immobilized probes on sensor chips, 5 μl of the DNA template were subjected to a double pre-amplification reaction before the unbalanced PCR to produce single-stranded target sequences useful for the interaction with the probes. In fact a double pre-amplification of templates was required, as already demonstrated in the application of Biacore™ system for the non-invasive prenatal detection of the fetal sex. The mutated probe was able to produce, with the target PCR product obtained from samples under analysis demonstrating for the first time the feasibility of this approach.

2.12. THALAMOSS AFTER THALAMOSS

2.12.1. The THALAMOSS Biobanks. Within THALAMOSS we demonstrated that freezing, cryopreservation and thawing steps maintain a steady erythroid differentiation potential of the cells in terms of both kinetics and types of hemoglobin produced. The validation of the cellular Thal-Biobank was consolidated by results obtained in other laboratories on different batches of the cryopreserved cells from the same patients. Also, these specimens provide an important opportunity for the research, to stratify patients based on all their phenotypic/genotypic characteristics, to evaluate the different ability of each individual to respond to inducers of HbF synthesis, and to develop novel therapeutic strategies for β-thalassemia. Correction of the biobanked cells by gene therapy and genomic editing is expected to be possible. Furthermore, generation of iPSCs is, at least in theory, a possible strategy. To our knowledge, the cellular Thal-Biobank described (Cosenza et al., 2016) is the only worldwide available cellular biobank for thalassemia, being a unique tool for theranostics of beta-thalassemia and (when implemented) of sickle-cell disease. The THALAMOSS partners involved in the development of the centralized cellular biobank are fully committed to maintain and implement this KEY feature of the applied research for thalassemia. The list of partners who will be responsible for this activity is the following (but the activity is open to collaboration by all the THALAMOSS partners and will be extended to other interested researchers/institutions): UNIFE, CING, LGHA, UNICA and will be coordinated by UNIFE. The search for international and local funding will be encouraged. The activity of this THALAMOSS activity will be:

- Search for additional funding sustaining the cellular biobank.
- Increase the collaborations of external researchers
- Expand the biobank to cells from sickle-cell disease (SCD) patients (a limited number of HbS patients are already present)
- Expand the biobank to cells for other hemoglobinopathies.

2.12.2. The Data set. One of the major results obtained by the project is the THALAMOSS Cohort data set, which has been collected using the THALAMOSS Data Management platform developed by MU (see Deliverable D4.3). The THALAMOSS Cohort has been established as a long-term resource and steps have been taken to make it findable and accessible according to the FAIR principles. For findability reasons, it has been included into the BBMRI-ERIC Directory 3.0 and newer, under collectionID of bbmri-eric:ID:CZ_MMCICollection:THALAMOSS. Accessibility and privacy has been implemented by establishing an access committee, as the data is pseudonymized and thus still considered personal. The access committee comprises of representatives of the data curator and all the THALAMOSS partners contributing the data into the data set: MU, UNIFE, CING, LGHA, UNICA, EMC, BRFAA. Activity of the access committee is instantiated after sending request to the data manager of the THALAMOSS Cohort (Ondřej Vojtíšek), as advertised in the BBMRI-ERIC Directory. Interoperability and reusability has been implemented by publishing the THALAMOSS Data Management architecture and documentation of the data model as a part of THALAMOSS D4.3. In addition collaborative efforts might be considered with other EU Project, such as RD-Connect (an EU project, see http://rd-connect.eu/) interested in multi-omics analysis of patients.
2.12.3. A specific issue that has been clarified by the THALAMOSS research is that the association between SNPs and inducer depends not only by the OMICS of the patients, but also by the inducer. NOVAMECHANICS will continue collaborating with CING, BRFAA and UNIFE to overview this issue and implement the information needed in order to stratify patients for response to HbF inducers, including those used in therapy. Towards the chemoinformatic analysis of HbF inducers, the classes of inducing agents are investigated with UNIFE (a) 34 analogues of trimethylangelicin already tested as HbF inducers on K562 cells as well as on erythroid precursor cells from erythroid precursor cells (ErPCs) from beta-thalassemic patients; (b) isoxazole derivatives shown to be among the best inducers of HbF found during the THALAMOSS project. This new class of potential HbF inducers will be tested in K562 cells and patient-derived ErPCs in order to select novel active analogues based on two isoxazole derivatives with alternative chemical skeletons, specifically 3,4-isoxazolidiamides and 4,5,6,7-tetrahydro-isoxazolo-[4,5-c]-pyridines. UNIFE and IRBM will continue in the possible further validation of possible therapeutic strategies based on combined treatments with HDAC inhibitors (patented by IRBM), rapamycin (Sirolimus) (patented by UNIFE).

2.12.4. The WEB site. The THALAMOSS activity dissemination suffers from the fact that, in spite of the very high number of publications (see D5.5) presentations to congresses, workshops, meetings (see D5.4) many data are still not public (for instance those presented in the final D3.2 D3.4 and D3.6 deliverables) and are expected to be considered for publication in the following months. Therefore it is of key interest for the THALAMOSS partners to maintain active the WEB-site for the next 5 year. To this aim UNIFE and BRFAA will collaborate to maintain the activity of the THALAMOSS WEB-site. Together with the Association for the Fight against Thalassemia, UNIFE and BRFAA will freely distribute the CD-ROM, which will be upgraded year-by-year including all the dissemination activity now present only in the WEB-site restricted area, but moving to the public area once the product will be published.

2.13. Data validation and quality control. UNIFE and BRFAA were involved in validation of transcriptomic data performed using RNA isolated from the same ErPCs allowed to differentiate with controlled experimental parameters. The two methodologies employed were RNA-seq (at BRFAA) and RT-PCR and microarray analysis performed using an Agilent platform (at UNIFE). The data obtained demonstrated that over 70% of up/down regulated genes were found by the two approaches. The response of ErPCs to HbF induction were analysed in parallel in cryo-stored samples by UNIFE and CU/CHOP obtaining the clear information that the haemoglobin pattern obtained independently in different laboratory is fully consistent. The quality control of the SNP polymorphisms have been conducted by UNIFE and UNICA on XmnI polymorphism, demonstrating a different result only in one case over the more that 200 samples analysed.

Potential Impact:
GENERAL INTRODUCTION. The concept developed by THALAMOSS and the expected results and deliverables were relevant to the topic of the call HEALTH.2012.1.2-1 (Development of technologies with a view to patient group stratification for personalized medicine applications). In particular, THALAMOSS has conducted research activities aimed at classifying β-thalassemia patients in clinically distinct subgroups (WP1, WP2). This has been done at unprecedented level. This specific part of the research will bring important information in the field of personalized medicine, i.e. tailored medical interventions which are more effective and have fewer adverse effects in specifically defined patient groups. This will bring important changes in patients’ attitudes and the need for molecular diagnoses. The possibility to perform diagnosis related to therapeutic strategy is expected to be of great value for the patients’ Associations in the next future. In fact, Guidance to personalized therapeutic interventions is achieved by the fact that THALAMOSS has developed diagnostic kits for patient stratification, also based on the fact that novel therapeutic strategies have been applied to cells isolated from β-thalassemia patients in order to correlate therapeutic efficacy with genotype and phenotype. The collaborations with SMEs are strictly linked with exploitation. In addition, the THALAMOSS results are relevant also for other sections of the HEALTH.2012. call, such 1.4-4 (Targeted nucleic acid delivery as an innovative therapeutic or prophylactic approach), 2.1.1-1 (Omic for rare diseases), 2.1.1-1-B (Clinical utility of -omics for better diagnosis of rare diseases), 2.1.1-1-C (Databases, biobanks and ‘clinical bio-informatics’ hub for rare diseases), 2.1.1-3 (Statistical methods for collection and analysis of -omics data), 2.4.4-1(Preclinical and/or clinical...
development of substances with a clear potential as orphan drugs), 2.4.4-3 (Best practice and knowledge sharing in the clinical management of rare diseases), 3.2-2 (New methodologies for health technology assessment).

3.1. Socio economic impact.

3.1.1. Scientific Impact. The scientific impact of the THALAMOSS Project was expected to be excellent, in view of the fact that, for the first time, genetists, clinicians, molecular biologists, experts in gene therapy and experts in the field of induction of fetal hemoglobin are included in a common Project aimed at solving very important points in basic as well as applied research on thalassemia. In fact, the impact of THALAMOSS on the scientific community society was of great relevance. According with deliverables D5.5 and D5.6 the scientific activity of THALAMOSS has been reported as lectures–oral presentations for a total number of 149 activities, most of which at international congresses and conferences. The total number of publications in peer-reviewed journals is 68, demonstrating the interest of the scientific community to the THALAMOSS results interestingly, an high percentage of these reports are published in very high impact factor journals, such as Cell, Blood, Nucleic Acids Res, Cell Reports, Nature Medicine, British Journal of Haematology. Moreover, it is reasonable to predict that at least 20-25 papers will be published in the next future, in consideration of the very high number of original observations and innovative results recently obtained and still unpublished (these are reported in deliverables D3.2 D3.3 D3.6 and D4.5). Finally, two special issues have been accepted and are under production, one focusing on medicinal chemistry for thalassemia (Novel options for the pharmacological treatment of hemoglobinopathies), the other focusing of theranostics (Theranostics of Rare Diseases).

These are examples of important scientific results obtained under THALAMOSS:

A. General

1. Recruitment of nearly 900 beta-thalassemia patients.
2. Validation of a protocol for cellular biobanking.
3. Production and characterization of the first cellular biobank for beta-thalassemia (at present the centralized cellular THALAMOSS biobank is constituted by more than 1340 cryovials from 213 patients.
4. Production of a THALAMOSS Cohort data set, which has been collected using the THALAMOSS Data Management platform and includes 663 patients and one cell line, 42 RNA-seq analyses and 9x6 proteomic analyses, and sequencing data coming from cell lines (overall size of omics data totals to 204GB).

B. Regulation of globin gene expression and identification of novel markers

5. Identification and characterization of a new Ay-Globin-Gene associated to high HbF production and efficient response to HbF inducers.
6. Demonstration of the crucial role for the ubiquitously expressed transcription factor Sp1 at early stages of hematopoietic specification.
7. Identification of mutations of KLF1 factor causing persistence of embryonic globin gene expression.
8. Identification of genomic variation in the MAP3K5 gene associated with beta-thalassemia disease severity and hydroxyurea treatment efficacy.
9. Demonstration that TAF10 Interacts with the GATA1 Transcription Factor and Controls Mouse Erythropoiesis.
10. Demonstration that ASH1L (a histone methyltransferase protein) is a novel candidate globin gene regulator.
12. Demonstration that KLF1 directly activates expression of the novel fetal globin repressor ZBTB7A/LRF in erythroid cells.
13. Demonstration that macrophages support pathological erythropoiesis in polycythemia vera and β-thalassemia.
14. Studies on the reactivation of developmentally silenced globin genes by forced chromatin looping.
15. Demonstration that forced chromatin looping raises fetal hemoglobin in adult sickle cells to higher levels than
pharmacologic inducers

C. Novel trends in therapy

17. Further development of known HbF inducers to be proposed for a clinical trial (rapamycin) and for patenting protection.
21. Demonstration that microRNA targeting can induce HbF production.

D. Diagnosis


3.1.2. Impact on Industry development. The impact of THALAMOSS research activity is reported in deliverable D7.9 (IPR Management Database: Final Release) and it is summarizes as follows. (a) A novel read-through corrector exhibiting high efficiency in inducing de-novo production of β-globin (described in detail in D3.4) has been identified. (b) Isoxazole derivatives which display a very high efficiency in inducing HbF. Moreover the HbF induction occurs by these compounds also in ErPCs not responsive to hydroxyurea. These data on the induction of fetal haemoglobin exerted by the compounds have been shared with Rare Partners. Based on the opinion of several members of the Steering Committee, it was confirmed that the patent application will be filed within the end of July 2017, protecting the findings toward a possible use in beta-thalassemia. (c) Innovative vectors for gene therapy of beta thalassemia and exhibiting very interesting efficiency when compared with already described therapeutic vectors (see D3.6). (d) Improved gene editing approaches for therapy of beta thalassemia exhibiting an high efficiency when compared with available protocols (see D3.6). These effects will be further verified and a decision will be taken to protect this invention, taking in careful consideration the complex patenting situation with respect to CISPR/Cas9 gene editing. Furthermore, a clinical trial has been approved based on Sirolimus (see deliverable D3.12: Market/business surveying and road mapping activity).

3.2. Wider societal implications of the project

3.2.1. Impact on the society. The impact of THALAMOSS on society is of great relevance. This is summarized in the deliverable D5.3 related to the dissemination of the project activities and results in popular print media. These include mainly newspapers, magazines, flyers and press releases. This deliverable targets a wide lay audience of all ages, including designers, engineers, scientists, researchers, teachers and students, and also associations of the patients and their relatives. The objectives were to promote a dialogue with the wider lay communities across Europe on thalassemia and related societal and ethical issues. In total 31 publications in popular print media took place. We like to underline that rare diseases, including thalassaemia, are being recognized as one of the major challenges for the health care industry, because it has a tremendous impact on health care systems as well as families of those affected by the diseases. Although each disease has a relatively low prevalence, there are more than 30 million of affected people in Europe and efforts to find new cures are considered as one of the main priorities. Just to give an example The International Rare Diseases Research Consortium (IRDRC; http://www.geneticalliance.org/irdrc) plans to deliver by 2020 diagnostic tests for most rare diseases, and 200 new therapies for patients affected by rare diseases. The possibilities offered by recent advancements in basic science in the field can be fully exploited. During the project execution, THALAMOSS has been in continuous contact with several Thalassemia Patients’
Associations, including the Cyprus Thalassemia Federation, the Cooley Anemia Foundation, the Thalassemia International Federation, the Veneta Association for the Fight Against Thalassemia, the Ferrara Association for the Fight Against Thalassemia. From all these organizations specific collaborations have been activated with the objective not only of disseminating THALAMOSS results, but only of collaborating in joint efforts to drive policy strategies on the field of rare diseases. Among the THALAMOSS results several issues deserves great attention from the patient’s Associations, in consideration of the fact that key results are of great impact for diagnosis and for therapeutic strategies. These are the main issues to be considered for the most important development in diagnostics and therapy: (1) Biobanking; (2) Gene analyses; (3) Personalized therapy; (4) Prenatal diagnosis; (5) Therapy: induction of fetal hemoglobin; (6) Gene therapy and combined therapies.

3.2.2. Impact of the THALAMOSS research on Biobanks.

Biobanking, iPSC and gene editing. Biobanking of biological material, including viable cells, is a new and very relevant approach which involves a wide range of public and private institutions. The number of biobanks is rapidly increasing, helping to create collaborative networks able to tackle very important biomedical issues needing of large numbers of tissue samples relating to the same groups of pathologies. In Europe, more than 600 biobanks and institutions from over 30 countries that collect samples and pathological/clinical data belong to the Biobanking and Biomolecular Resources Research Infrastructure (BBMRI-ERIC). While biobanking of biological material (cellular pellets, DNA, RNA) is important to organize collection of high-quality samples with reliable clinical information for diagnostics, therapy and research, the output is limited by the fact that experiments with biological modifiers cannot be performed. On the contrary, this is possible, at least in theory, with validated cellular biobank. As we pointed out in the deliverables D1.6 and D1.7 cellular biobanking is a key step in “OMICS” analyses, as well as in the screening of bioactive molecules for the development of novel therapeutic protocols. The patients associations should carefully follow this issue, since it is expected that induced pluripotent stem cells will be generated from cryopreserved cells, generating a second and extremely interesting beta-THAL-iPSC-Biobank, that could be use for correcting the mutations by gene editing using approaches based on ZFN, TALEN, and CRISPR-CAS9, which comprise specifically engineered DNA binding domains fused to nuclease. The combination of HSC (hematological stem cells) and iPSCs (induced pluripotent stem cells) production with gene correction strategies appears one of the most promising approaches for genetic diseases, including hematological pathologies, such as β-thalassemia and sickle-cell disease. Notwithstanding this apparent setback, iPSCs are a promising substrate for gene therapy, as they can be amplified in vitro indefinitely (where they are still subject to the same mutation rates and potentially undesirable changes as any other cell type) and thus allow the clonal selection of rare events of therapeutic interest. Since their inception, iPSCs have therefore been used extensively in novel therapies for β-thalassemia and other haemoglobinopathies, as will be detailed for specific GT approaches below.

Gene editing on iPSC: the choice of the strategy. The relative benefits of alternative gene editing systems is at present under debate and should be carefully monitored with respect to several parameters, of which the most important are efficiency and off-target effects. The most employed genome editing system to the iPSCs is the CRISPR/Cas9 strategy, but other approaches are available, such as those based on zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs). Moreover, other Cas9-like systems have been described, including the CRISPR/Cpf1 nuclease platform, dimeric RNA-guided FokI nucleases, and use of Cas9’s derived from a variety of prokaryotic species.

Gene editing: the choice of the genomic target. It is expected that this approach will be useful for any β-thalassemia mutations, with the exception of large gene deletions in which globin gene addition (instead of globin gene correction) could be considered. While the CRISPR/CAS gene correction has been usually directed to the β-thalassemia mutation, the described strategy might also consider targeting of other genomic region involved in key processes, such as production of fetal hemoglobin and generation of a HPFH-like phenotype. It should be underlined that genome editing-based therapies rely not only on gene correction but also on the disruption of target gene regions. In the case of β-thalassemia this targeting might include not only discrete regions of the β-globin gene cluster, but also transcriptional repressors of the γ-globin gene, such as BCL11A and LRF/ZBTB7A.
CRISPR/Cas9 and iPSC technologies: from the laboratory to the clinic. Despite the promising developments of CRISPR-based methodologies, many challenges have to be overcome before the system can be applied therapeutically in human patients. Enabling delivery technology is one of the key challenges. With respect to consideration on clinical trial, ZFNs are the editing technology which has been considered for clinical trials in several protocols fighting several pathologies. However, recent developments concern also the CRISPR/Cas9 approach, which the basis for the proposal of four clinical trials based on CRISPR and focusing on the use of PD-1 knockout engineered T cells for metastatic non-small cell lung cancer (NCT02793856) and renal cell carcinoma (NCT02867332), hormone refractory prostate cancer (NCT02867345) and invasive bladder cancer Stage IV (NCT02863913). In these clinical trials CRISPR will be used to neutralize the PD-1 gene, which expresses a protein on T-cell surfaces that many cancers can turn off, thereby blocking T-cell antitumor attacks. Once expanded in the laboratory, the edited cells will be returned to the patient. The engineered cells will circulate and hopefully home in on the cancer site. The use of iPSCs in clinic is the object of a limited number of trials (more than 15 in clinicaltrials.gov).

Comment: The gene editing approach cover almost all of the mutations causing β-thalassemia. The patients should require to know their gene pattern, including, but not limited to, the primary mutations affecting the β-globin gene cluster.

3.2.3. Gene analyses: Characterization of known SNPs in a selected patients cohorts.

The following is an example of the data we could generate using the THALAMOSS Data Set. The Data Set allows comparisons between selected populations, facilitating the identification of suitable biological markers predicting the severity of the disease on one hand and the response to therapy on the other. The knowledge of the primary mutation as well as the associated SNPs is relevant in consideration of the fact that these gene analysis can predict the clinical severity of the disease as well as the response to HbF inducers (see also deliverable D4.5). Therefore it is very important that these analysis will be done (in the THALAMOSS data set platform all these analysis are present).

For example, Several DNA polymorphisms have been associated with high production of fetal hemoglobin (HbF), although the molecular basis is not completely understood. In order to identify and characterize novel HbF-associated elements, we focused on five probands and their four families (from Egypt, Iraq and Iran) with thalassemia major (either β(0)-IVSI-1 or β(0)-IVSI-1) and unusual HbF elevation (>98 %), congenital or acquired after rejection of bone marrow transplantation, suggesting an anticipated favorable genetic background to high HbF expression. A polymorphism of the Ay-globin gene is here studied in four families with β(0)-thalassemia (β(0)-IVSI-1 and β(0)-IVSI-1) and expressing unusual high HbF levels, congenital or acquired after rejection of bone marrow transplantation. This (G→A) polymorphism is present at position +25 of the Ay-globin genes, corresponding to a 5'-UTR region of the Ay-globin mRNA and, when present, is physically linked in chromosomes 11 of all the familiar members studied to the XmnI polymorphism and to the β(0)-thalassemia mutations. The region corresponding to the +25(G→A) polymorphism of the Ay-globin gene belongs to a sequence recognized by DNA-binding protein complexes, including LYAR (Ly-1 antibody reactive clone), a zinc-finger transcription factor previously proposed to be involved in down-regulation of the expression of γ-globin genes in erythroid cells. We found a novel polymorphism of the Ay-globin gene in four families with β(0)-thalassemia and high levels of HbF expression. Additionally, we report evidence suggesting that the Ay-globin gene +25(G→A) polymorphism decreases the efficiency of the interaction between this sequence and specific DNA binding protein complexes.

Comment: The knowledge of the primary mutations causing β-thalassemia and of the polymorphisms of the disease modifiers is of relevance for the β-thalassemia patients and for the clinicians, since it appears that patients might be stratified with respect to these genetic parameters. Stratified patients might be differentially treated on the road of precision medicine.

3.2.4. The THALAMOSS Cohort data set.
One of the major results of THALAMOSS activity is the THALAMOSS Cohort data set, which has been collected using the THALAMOSS Data Management platform developed by MU (see Deliverable D4.3). As of the end of the THALAMOSS project, the cohort includes 663 patients and one cell line, 42 RNA-seq analyses and 9x6 proteomic analyses, and sequencing data coming from cell lines (overall size of omics data totals to 204GB). To maintain its impact even after the end of the THALAMOSS project, the THALAMOSS Cohort has been established as a long-term resource and steps have been taken to make it findable and accessible according to the FAIR principles. For findability reasons, it has been included into the BBMRI-ERIC Directory 3.0 and newer, under collectionId of bbmri-eric:ID:CZ_MMCI:collection:THALAMOSS. Accessibility and privacy has been implemented by establishing an access committee, as the data is pseudonymized and thus still considered personal. In addition collaborative efforts might be considered with other EU Project, such as RD-Connect (an EU project, see http://rd-connect.eu/) interested in multi-omics analysis of patients. The THALAMOSS project is a suitable example RNA-seq and proteomics data linked to the clinical data of the thalassemic patients organized in a unique combination.

### 3.2.5. Target the gene: potential avenues leading to precision medicine for β-thalassemia.

In consideration of the fact that β-thalassemias are characterized by several different gene mutations, they can be considered for effective personalized therapy on the road of precision medicine. Based on the available data, the mutations causative of β-thalassemia (examples are reported in Figure 1) are different and can be divided in (a) gene deletions, (b) alteration of the β-globin gene promoter, (c) point mutations affecting the RNA translation and including frameshift mutation, sense mutations and mutations occurring at ATG initiation codon, (d) alterations of functional splicing sites, (e) activation of cryptic splicing sites, (f) alteration of the polyA site. Of course, each mutation requires specific therapeutic interventions, if a personalized therapeutic approach is being considered.

**Pharmacological correction of abnormal splicing sites**

Despite mutations causing beta-thalassemia are many, only 10 mutations are responsible for the majority of cases worldwide and some of the most frequent cause aberrant splicing of intron 1 (IVS1-110, IVS1-6, IVS1-5) or intron 2 (IVS2-654, IVS2-745). These mutations lead to incorrectly spliced mRNAs, even though the correct splice sites remain undamaged and potentially functional. Use of small nuclear RNA (snRNA) and splice-switching oligo-nucleotides represents a promising approach since these molecules can restore the corrected splicing re-establishing the synthesis of the normal protein. Therefore blocking the aberrant splice sites with antisense oligonucleotides forces the splicing machinery to reselect the existing correct splice sites. Expression of antisense sequences targeted to the aberrant splice sites in thalassemic pre-mRNA has been successful, restoring the correct splicing pattern and ultimately restoring hemoglobin synthesis. This was demonstrated in HSCs and erythroid progenitor cells from a patient with IVS2-745/IVS2-1 thalassemia. After transduction of the patient cells with a lentiviral vector that express an snRNA targeting the mutant RNA, the levels of correctly spliced β-globin mRNA and adult hemoglobin were approximately 25-fold over baseline. Similarly, the correct splicing pattern was restored in a mouse model of IVS2-654 thalassemia. This was achieved by delivery in vivo of a splice-switching oligonucleotide, a morpholino oligomer conjugated with an arginine-rich peptide. Repaired β-globin mRNA restored significant amounts of hemoglobin in the peripheral blood of the IVS2-654 mouse, improving the number and quality of erythroid cells.

**Readthrough molecules for stop-codon mutations**

Another approach showing a great potential for the treatment of genetic disorders characterized by to premature termination codons (PTCs) is the use of drugs to induce stop codon readthrough. These modified RNA would protect against nonsense-mediated mRNA decay (NMD) and allow production of a protein. Aminoglycoside antibiotics can decrease the accuracy in the codon-anticodon base pairing, inducing a ribosomal read-through of PTC. Aminoglycosides act upon binding to the 18S ribosomal subunit, subverting normal ribosomal DNA proofreading activity to permit the incorporation of an alternative amino acid at the post-transcriptional control (PTC) level. Aminoglycosides and analogous molecules were tested in their ability to...
restore β-globin protein synthesis on human erythroid cells (K562) carrying a lentiviral construct containing the β0-39 globin gene. Treatment of these cells with Geneticin (G418) and other aminoglycosides restored the production of β-globin. Moreover, after FACS and high performance liquid chromatography (HPLC) analyses, G418 was also demonstrated to partially correct the biological function of the β0-39 globin mRNA in erythroid precursor cells from β0-39/β0-39 homozygous thalassemia patients. This study strongly suggests that ribosomal read-through should be considered a novel approach for treatment of thalassemia caused by premature stop codon mutations and NMD. Other compounds that should be considered are the non-aminoglycoside ataluren (PTC124) has up to 15 times more potency than other read-through agents on mRNAs carrying stop codon mutations. Moreover, the anti-inflammatory agent amlexanox has dual capabilities as NMD inhibitor and inducer of PTC read-through. Unfortunately, the read-through approach was found to exhibit low efficiency on thalassemia cells. As we report in deliverable D3.4 novel potent readthrough molecules have been found under THALAMOSS, on a subset of patients.

3.2.6. Diagnostics: novel approaches for personalized therapy and non invasive prenatal diagnosis.

Biological tests and genetic analyses for diagnosis and characterization of hematological diseases in health laboratories should be designed and developed with the aim of meeting the major medical needs of hospitals and pharmaceutical companies involved in this field of applied biomedicine. Basically, biological tests involve adult/newborn subjects, while genetic analyses involve adult thalassemia patients, newborns, embryos/fetuses, pre-implantation embryos and pre-fertilization oocytes. Genetic testing approaches to perform diagnosis consist of molecular techniques, that should be absolutely reproducible, fast, sensitive, cheap, portable. For instance, polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) are the two common techniques employed on a single or two cells obtained via embryo biopsy during pre-implantation diagnosis. They are useful mainly for diagnosis and/or prognosis, even though recent studies point out the need of genetic information for the development of personalized therapy.

Since the discovery of cell-free fetal DNA (cffDNA) in maternal plasma, diagnostic non-invasive prenatal methods have been developed or optimized for fetal sex determination and identification of genetic diseases. As far as fetal sex determination, this might be important for therapeutic intervention on sex-associated pathologies such as Duchenne muscular dystrophy, hemophilia and congenital adrenal hyperplasia. Surface plasmon resonance (SPR)-based biosensors might be useful for these studies, because they allow to monitor the molecular interactions in real-time providing qualitative and quantitative information, through kinetics, affinity and concentration analyses.

Within THALAMOSS the activity on molecular diagnosis was focused on three issues: (a) demonstrate the feasibility of SPR-based biosensors for real-time detection of Y-sequences; (b) develop pre-natal diagnostic approaches for β-thalassemia; (c) verify whether SPR-based prenatal diagnosis can be applied to β-thalassemia.

In a first study the Biacore™ X100 has been applied to identify Y-chromosome sequence in cffDNA obtained from plasma samples of 26 pregnant women at different gestational ages. We have demonstrated that there is a statistically significant difference between samples collected by pregnancies carrying male or female fetuses. Moreover, cffDNA obtained at early gestational ages and not detectable by conventional quantitative real-time PCR can be discriminated with high accuracy and reliability using SPR-based biosensors.

In a second study Four TaqMan® genotyping assays for the most common β-thalassemia mutations (β039, β+IVSI-110, β+IVSI-6, β0IVSI-1) present in the Mediterranean area were designed and validated for the genotype characterization of genomic DNA extracted from 94 subjects comprising 25 healthy donors, 33 healthy carriers and 36 β-thalassemia patients. In addition, 15 specimens at late gestation (21-39 gestational weeks) and 11 at early gestation (5-18 gestational weeks) were collected from pregnant women, and circulating cell-free fetal DNAs were extracted and analyzed with these four genotyping assays. These genotyping assays are able to detect paternally inherited point mutations in the fetus and could be efficiently employed for non-invasive prenatal diagnosis of β-globin gene mutations, starting from the 9th gestational week.
In the third study we have focused on the possible application of the developed SPR-based techniques as a non-invasive diagnostic tool to identify genetic point mutations such as the β+ IVSI-110 responsible of a β+ thalassemia phenotype. This non-invasive diagnostic strategy for the detection of the β+ IVSI-110 thalassemia mutation inherited from the father, was applied to blood samples obtained from pregnant women at different gestational ages. The mutated probe was able to produce, with the target PCR product obtained from samples under analysis demonstrating for the first time the feasibility of this approach.

3.2.7. Therapy: induction of fetal hemoglobin: from the pre-clinical data to a clinical trial.

Induction of fetal hemoglobin (HbF) is one of the approaches expected to bring new hopes in the treatment of β-thalassemia patients. This is based on the observation that β0-thalassemia patients with an HPFH (Hereditary Persistence of Fetal Hemoglobin) phenotype display milder clinical parameters. Several laboratories have confirmed in independent in vivo trials that the treatment of β-thalassemia patients with the HbF-inducer hydroxyurea (HU) leads to a clear improvement of the blood-intake requirements and even transfusion-independency in about 40-60% of the cases. Following this research efforts have aimed at the identification of novel HbF inducers with higher efficiency, lower toxicity and bone marrow suppression in comparison to HU. The search for HbF-associated polymorphisms has recently focused on stratifying β-thalassemia patients with respect to response to expectancy of the first transfusion, need for annual intake of blood, response to HbF inducers the most studied of which is hydroxyurea. Recent published studies demonstrate the possibility to predict response to hydroxyurea of β-thalassemia patients. This possibility is of interest for patients, as well as clinicians involved in patient management. There is no question that independence from blood transfusion is, together with the prevention of extramedullary hematopoiesis and ineffective erythropoiesis, a major objective in the management of β-thalassemia patients. This especially relevant in developing countries in which blood is scarcely available and often contaminated. Hence this field is one of the most interesting for the development of orphan drug products.

Several fetal hemoglobin inducers are available. All the HbF inducers are reported in the scientific literature have patents and clinical trials ongoing. Examples of patents applications linked to orphan drugs are those based on HQK-1001 (2,2-dimethylbutyric acid; patent US8242172), sodium phenilbuyrate (patent EP1162884), arginine butyrate (patent US7910624), 2-dimethylbutyrate (patent US8618068) and isobutyramide (patent US5439939). Examples of clinical trials employing orphan drugs acting as HbF inducers are NCT00790127 (Phase 1/2 Study of HQK-1001 in Patients with β-Thalassemia) and NCT00006136 (Phase II Study of Ariginie Butyrate With or Without Epoetin Alfa in Patients With Thalassemia Intermedia).

Drug repurposing and similar terms (‘drug repositioning’, ‘drug repprofiling’, ‘drug redirecting’, ‘drug rescue’, ‘drug re-tasking’ and/or ‘drug rediscovery’) have been coined for the development of existing medication for new indications in rare diseases together with the off-label use of pharmaceutical products under clinical trial regimen. The advantage of drug repurposing over traditional drug development is that the repositioned drug has already passed a significant number of short- and long-term toxicity tests, and pharmacokinetic and pharmacodynamic (PK/PD) studies. As the safety of these drugs is known the risk of failure to come to market is significantly reduced. Repurposed drugs can cut down the time needed to bring a drug to market. Patent filing of repurposed drugs is expected to catch the interest of pharmaceutical industries interested to the development of therapeutic protocols for RDs.

One example is rapamycin (sirolimus), which is designated as an orphan drug for treatment for chronic non-infectious uveitis (EU/3/11/898) and prevention of arteriovenous access dysfunction in patients undergoing surgical creation of an arteriovenous access for hemodialysis (EU/3/13/1204). However we have demonstrated that rapamycin is a strong inducer of fetal hemoglobin. They studied the effects of rapamycin on cultures of erythroid progenitors derived from the peripheral blood of 10 β-thalassemia patients differing widely with respect to their potential to produce HbF. They employed a two-phase liquid culture procedure for growing erythroid progenitors, high performance liquid chromatography for analysis of HbF production and reverse transcription polymerase chain reaction for quantification of the accumulation of globin mRNAs. The results
demonstrated that rapamycin induced an increase of HbF in cultures from all the β-thalassemia patients studied and an increase of their overall Hb content/cell. The inducing effect of rapamycin was restricted to γ-globin mRNA accumulation, being only minor for β-globin and none for α-globin mRNAs. This conclusion was recently confirmed by Pecoraro et al. (Hemoglobin, 2015, 39, 225-229) In this study they evaluated the efficacy of rapamycin in cultured erythroid progenitors from 25 sickle cell disease and 25 β-thalassemia intermedia (β-TI) patients. The results of the study demonstrated an increase in vitro of γ-globin mRNA expression in 15 sickle cell diseases and 14 β-TI patients and a corresponding Hb F increase. The induction by rapamycin, even if lower or similar in most of samples analyzed, in some cases was higher than hydroxyurea, supporting the concept that rapamycin could be a good candidate to be used in vivo for the treatment of hemoglobinopathies. Patents are available (EP1521578, “A novel use of rapamycin and structural analogues thereof” and US7541380, “Use of rapamycin and structural analogues thereof”) for the use of rapamycin for β-thalassemia.

Overall concept underpinning the project and background information on the product. The idea which is the basis of the pilot clinical trial (SIR-for-THAL) is that the use of Sirolimus, an immunosuppressant drug, for beta-thalassemia is that it is capable to increase HbF. Overall, available data indicate that: (a) Sirolimus increases HbF in cultures from beta-thalassemia patients with different basal HbF levels; (b) Sirolimus increases the overall Hb content/cell; (c) Sirolimus selectively induced gamma-globin mRNA accumulation, with only a minor effect on beta-globin and no effect on alpha-globin mRNAs; (d) there is a strong correlation between the increase by Sirolimus of HbF and the increase in gamma-globin mRNA content. Taken together, these data suggest that Sirolimus may be an interesting candidate for a role in the treatment of patients with beta-thalassemia. Thus we suggest, on the basis of the available evidence, to test Sirolimus in beta-thalassemia patients, having as a tentative indication “Treatment of beta-thalassemia intermedia and major”, using a dosage lower or, at maximum, equal to the ones already used for other indications. The tested product is already available in many European countries with the Trade Name Rapamune, indicated in transplant recipients. The Investigational Medicinal Product (IMP) will be provided as finished product by the Company who produces Rapamune, namely Pfizer. No development has been performed in terms of toxicology since, as previously mentioned, the product is already on the market with different indications.

3.2.8. Combination therapy based on gene addition, gene editing and HbF inducers. As already pointed out, induction of endogenous fetal hemoglobin (HbF) is one on the most widely applied therapeutic strategies for β-thalassemia and sickle-cell anemia (SCA) (4). While most of the recent studies in the field still focus on small-molecular-weight HbF inducers in isolation, lately the innovative strategy of combining vector-derived β-globin with the induction of endogenous HbF has been investigated. The combined treatment induces an increase of both, HbA (by gene addition) and HbF (by chemical HbF induction), with important therapeutic implications, given that gene augmentation in β-thalassemia major has been unable to reach physiological levels of β-like globin to date and might thus only lead to partial phenotypic correction. Since increased production of HbF is beneficial in β-thalassemia, the one-off application of gene therapy substituted with chronic application of HbF inducers appears to be a pertinent strategy to achieve clinical benefits not achievable with either strategy alone. When gene therapy was combined to HbF induction, the results obtained demonstrate that this combination strategy achieves high levels of functional haemoglobin in β-thalassaemic cells and a concomitant sharp decrease of excess α-globin, with significant scope for further improvements for what is as yet a nascent field of research. This strategy, at least in theory, also applies to gene editing and HbF induction.

Final Comment. Beta-thalassemias are a group of hereditary hematological diseases caused by more than 200 mutations of the human β-globin gene, leading to low or absent production of adult β-globin and excess of α-globin content in erythroid cells, and causing ineffective erythropoiesis and low or absent production of adult hemoglobin (HbA). More than 300,000 children are born each year with severe homozygous states of these diseases. It is estimated that about 7% of the world’s population is a carrier of a hemoglobin disorder. Together with sickle cell anemia (SCA), the thalassemia syndromes are the most important problems in developing countries, in which the lack of genetic counselling and prenatal diagnosis have contributed to the maintenance of a very high frequency of these genetic diseases in the population. This contributes significantly to driving changes in the distribution of carriers and affected people in relation to the migration of populations from endemic areas to countries where their prevalence in indigenous populations had been traditionally low (USA, Canada,
Australia, South America, the United Kingdom, France, Germany, Belgium, the Netherlands and, more recently, Scandinavia). These deep changes have encouraged most of the health systems of these countries in facilitating access to the prevention and treatment services available for these hemoglobin disorders. On the other hand, considering limitations and side effects of the currently available therapeutic approaches and management of the thalassemia patients, novel alternative options for therapy are urgently needed.

List of Websites:
http://thalamoss.eu/

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