Final Report Summary - TB PAN-NET (Pan-European network for the study and clinical management of drug resistant tuberculosis)

Executive Summary:
Drug-resistant tuberculosis strains is jeopardising TB control worldwide. Over 450,000 new cases of multi drug resistant TB (MDR-TB) occur each year and the prevalence of MDR-TB cases present in the world today may approach or exceed 1 million cases. About 10% of MDR-Tb cases are also resistant to the two most important classes of second line drugs (XDR-TB). Management of the DR TB cases is challenging and is associated to high costs and very poor outcome. Because of the very limited therapeutic options, the global threat of XDR-TB has major implications for public health. This project addressed some of the critical issues involved in MDR-TB management and control such as identification of determinants of Drug Resistance (DR) in different European settings and effective ways to link an improved diagnosis of DR to an effective treatment. The integration of basic science, focusing on standardization and improvement of lab diagnosis, and clinical science will provide a unique way to link the two fields and help the TB control community to better understand and treat the DR-TB cases.
quality drug susceptibility testing, small and medium-sized enterprises (SMEs) and expert clinicians has created the optimal background to generate new basic knowledge and novel more effective diagnostic tools (GenoType® MTBDRsl version 2.0). These tools include new platforms for diagnosis of TB and DR-TB, new tools for monitoring the response to treatment and innovative biomarkers for TB infection and disease.

We showed that serum miRNAs should be considered as promising biomarkers for the diagnosis of different TB stages and for monitoring progression of the disease. New diagnostic molecular tests able to discriminate live versus dead bacteria, suitable for treatment monitoring, have been investigated. We showed that quantitative molecular techniques amplifying nucleic acids from vital bacteria could be an alternative for monitoring early treatment response and for preliminary evaluation of personalized regimens. We studied the transcriptional regulatory (TR) network of M. tuberculosis in sensitive and resistant strains (including strains with different genetic background) under stress conditions. Different parameters were evaluated including survival to the selective stress and transcriptional response, in order to study the 'cost' in terms of bacterial fitness of the acquisition of the MDR phenotype. SmallRNAs in M. tuberculosis have been identified for the first time and full transcriptome (messenger RNAs and smallRNAs) of M. tuberculosis during stress response is now available.

A panel of M. tuberculosis strains mono-resistant to selected second line drugs has been developed and used for quality assessment purposes contributing to the standardization of quality of laboratory diagnosis at EU level. Among the major achievements of this project we can mention the first attempt to classify mutations in pncA gene based on capacity to confer resistance to pyrazinamide. A large web-based database for collection of clinical, epidemiological and molecular data has been set up and made available on the project web site.

Several multi-centre studies have been completed and others on-going across Europe, including (1) retrospective analysis of MDR/XDR TB risk factors, survival and HIV co-infection rates; (2) multi-centre evaluation of Hain Lifesciences GenoType® MTBDRsl test and feasibility study of the MDR/XDR-TB Color drug susceptibility testing in M. tuberculosis strains (including the Color Test performance evaluation on prospectively collected sputum samples, and further development of the test for DST of unconventional second-line drugs); (3) evaluation of GenoType® MTBDRplus performance; (4) novel biomarkers for TB disease and cure (5) detection of mutations relevant for the prediction of PZA resistance. Capacity building and training across EU were considered a priority in the project. We built capacity to perform clinical and diagnostic trials in four sites in TB endemic Countries by providing infrastructures, human resources capacity and specific training. An innovative training package on DR-TB management was developed and used to train a relevant cadre of professional across Europe. Training modules and educational material for “training of facilitators” for post graduate courses were developed. This material, included in the first version of the Pulmonary TB chapter of the ERS Handbook (ERS-HERMES project), is continuously updated and further evaluation of training material has been initiated to ensure up-to-date material within the planned courses. Project dissemination activities have been ensured through various events such as ERS general meetings, symposia, training courses as well as publication of articles about the TB PAN-NET project in the ERS Newsletter, distributed to over 30,000 clinicians across Europe. The project website, available also in main Eastern European languages (Hungarian, Polish, Romanian and Russian), is regularly maintained and implemented to contribute to project promotion through the advertisement and highlights of its activities. The website also serves as collaboration system for Project Partners.
Project Context and Objectives:

TB PAN NET is the acronym given to a major European translational research consortium of 28 partners with the aim to address the challenge of multidrug resistant tuberculosis (MDR TB) that faces Europe by establishing a network of expert partners who possess extensive experience in the conduct of basic and clinical research related to MDR TB, TB control and molecular epidemiology. The project was divided into 8 workpackages, 1 organising and 7 scientific.

The main objective of WP1 was the characterization of the genotypic profile of all M. tuberculosis strains including MDR, isolated in a network of selected metropolitan settings in Europe (London, Milan, Hamburg, Stockholm, Bruxelles, Riga, Tartu and Vilnius), by international standardized molecular methods (automated MIRU-VNTR typing and spoligotyping) and the evaluation of the epidemic spreading of drug-susceptible/ drug-resistant TB by cluster analysis. Training of WP1 participants to automated MIRU-VNTR was planned, ensuring quality-controlled and standardised use by the partners of workpackage, which is essential for consistence and reliability of this multicentre study. The WP1 was also aimed to:

- Identify clinical and social risk factors (immune-depression, immigration, poverty, homelessness, detention) associated with the development of drug-resistant TB in the selected European cities, including a comparison among the different metropolitan settings,
- define TB transmission paths, identifying both unsuspected risk factors associated with TB transmission
- describe transmission networks

The development of an easy-to-use TB surveillance system that combines a web based service for the automated identification of M. tuberculosis strain types in combination with clinical data such as drug resistance and epidemiologic data (time, place, person), was another objective of WP.

The WP2 was aimed to deep understand the molecular mechanisms responsible of the emergence of resistance to 1st line and in particular 2nd line drugs recently introduced in MDR- and XDR-TB treatment. The WP2 produced a list of validated mutations affecting genes encoding putative and well-known targets for anti-TB drugs, characterizing among others the pncA gene leading to resistance to the key drug pyrazinamide, with a large study including structural analysis and list of mutations divided for their capacity to predict DR to the drug. The work was also dedicated to the evaluation of the role of compensatory mutations in affecting the fitness of DR strains as well as to develop databases for high- and low-confidence genetic markers of resistance to rifampicin, ethionamide, fluoroquinolones and second-line injectable drugs. Considering the need of more advanced, robust and easy-to-perform assays for DR-TB diagnosis, the WP2 developed suitable “home-made” techniques to provide proof-of-concept for further improvement of existing molecular diagnostic tools for TB, MDR- and XDR-TB detection. A propidium monoazide (PMA) assay, suitable for monitoring patients under treatment that selectively amplify the nucleic acids from only live bacteria was validated. A rapid test for XDR-TB detection (GenoType®MTBDRsl version 2.0) was optimized and novel microarray-based assays were developed for MDR- and XDR-TB diagnosis. Furthermore, circulating mRNA were identified as biomarkers for TB diagnosis.

In addition the WP2 aimed at studying new regulatory mechanisms involving non-coding RNAs: the transcriptional regulatory network in MTB was implemented to study the correlations of resistant phenotypes and virulence features. Small non-coding RNAs were discovered as involved in antibiotic and stress response to MTB and DR-associated SNPs were found in intergenic regions matching with sRNA, thus providing new information on the role of regulatory pathways in drug resistance phenotype.

Finally the antimycobacterial activities of new compounds (heterocyclic compounds, para-aminosalicylic...
Octahydrophenanthridinediones) were evaluated and represent promising agents for treatment of TB.

The EQA for DST for 1st and 2nd line drugs has been established and published. Part of the WP3 has been dedicated to the collection of resistant and susceptible M. tuberculosis, including lab-generated strains with mono-resistance useful for external and internal quality control. Part of the WP was also dedicated to provide information on the most suitable method to detect hetero-resistance, showing that conventional drug susceptibility testing is better suited than the tested molecular methods to detect heteroresistance. The WP also showed that different decontamination procedures and storage conditions have little impact on the detection of heteroresistance as well as it was demonstrated that the sensitivity of RMP resistance detection depends on the method used.

The principal aim of WP4 was to establish the infrastructure and technical capacity for an EU-wide network capable of developing and evaluating new diagnostic systems conducting multicenter clinical trials of novel drugs for the treatment of MDR-TB. This would be achieved through the development and implementation of harmonised ethical, financial, governance, laboratory and clinical protocols and practices. Ultimately this network would be a key EU resource to develop best-practice clinical management strategies for MDR/XDR-TB, to address key public health issues and would be seen by the EU and international diagnostic and pharmaceutical companies as a “first-stop” for the development and evaluation of new products.

This principle aim was achieved through:
- development of a training curriculum and establishing a laboratory training programme to improve the laboratory performance and develop a cadre of new European microbiologists with expertise in TB and mycobacterial diagnosis and analysis;
- conducting a series of multicentre demonstration and evaluation studies using them as a mechanism for the development and implementation of common protocols, quality systems, laboratory and clinical training;
- recruitment and follow-up of two longitudinal cohorts of sensitive and MDR-TB patients, establishing their baseline characteristics, risk factors for MDRTB, rates of HIV co-infection and survival followed by a development and comprehensive evaluation of novel proof-of-cure host and pathogen biomarker assays which will be assessed against existing gold standard methods including microscopy and bacteriological culture. New biomarkers would enable us to determine the point at which patients cease to be infectious as early as possible more precisely, thus improving treatment outcomes, infection control and reducing institutional cross-infection. It would help to determine whether a treatment regimen was well chosen and effective and establish the earliest point at which an individual was cured bacteriologically and how this correlated with clinical, radiological and immunological markers of improvement.

The principal aim of WP5 was to provide quality education and training on TB drug resistance and in particular on MDR/XDR-TB for TB programme managers at national and sub-national level, responsible for planning, organizing, implementing and evaluating activities within tuberculosis (TB) control programmes and/or HIV/AIDS programmes, and for Western and Eastern European clinicians in order to develop the human resources on TB and thus curb the drug-resistant TB epidemic. This was achieved through the development of training modules based on the international standards and requirements on clinical management and control of MDR-TB. Clinical and operational research are considered crucial by international organisations for improving control of TB thus a dedicated module was developed, field tested, revised in collaboration with P13 after the first pilot course and made available for use for health
care training at the selected study sites according to the ToT (Training of Trainers) approach. This principal aim was achieved through the development of different training material:
- training modules that will target the health staff at regional and national level and in particular managers of national/sub-national TB and TB/HIV programmes, agencies and NGOs involved in MDR-TB programmes in Europe with special focus on Eastern European countries and globally;
- training material for respiratory clinicians and specialists with specific aim to prepare specialists from Eastern Europe and former Soviet countries, who afterwards will be able to carry out quality medical practice in their countries and to train others in how MDR/XDR-TB patient management needs to be done. All the material was developed in modules easily adaptable to different settings and yearly updated and revised. All the material developed will be available to the requesting NTP for local adaptation.
Within this work package the P6 (ERS) played a key role as its aim is to harmonise and promote the highest standards of practice in its field of respiratory medicine targeting European clinical specialists. A panel of Post-Graduated (PG) courses based on the MDR/XDR modules and materials developed for TB programme staff, readjusted for a complementary target (Western and Eastern European specialists) were offered during the ERS Annual Congress. The ToT (Training of Trainers) approach was used in ERS PG courses, External School courses and seminars allowing the delivery of a quality and standardized training relying on a pool of qualified facilitators.
The aim of WP 6 was to establish clinical research in drug resistant tuberculosis in Europe, using a clinical database. We were seeking for a detailed description of treatment outcomes in individual cases and important factors (risk factors, comorbidities, resistance patterns, adverse events, drugs, dosing, treatment monitoring) which affect the outcome in various European settings. The investigation compared patients with non- multidrug-resistant TB with patients with multidrug-resistant tuberculosis. Cost of treatment should also be evaluated. The information gathered should inform policy makers and clinicians in order to take appropriate decisions regarding general disease control measures, but also individual treatment decisions.
Contextual factors of treatment of drug resistant, like delayed diagnosis, slow resistance testing and extremely long treatment duration from in average 24 months at different treatment centers are particular challenges in investigating patients with drug resistant tuberculosis.
The question was approached
- by a literature review and the development of a questionnaire, targeting important questions in the management of drug resistant tuberculosis in Europe.
- by development of an electronic and paper data collections tool
- by role out and implementation of the study at participating institutions after ethical approval, using face to face training and guidance by investigators manuals
- inclusion and follow up of the maximum possible number of cases at each participating side - until a definite treatment outcome was achieved
- ongoing analysis, interpretation and publication of results after completion of treatment in 380 cases of multidrug-resistant tuberculosis and 376 cases of non-multidrug resistant tuberculosis
- by collection of data on drug costs in all EU countries and analysis of cost of treatment, regarding the use of 2nd line drugs in multidrug-resistant tuberculosis.

Final evaluation is ongoing and will continue beyond the end of the TBPANNET funding period, as treatment outcomes of the cohort could only be obtained at the end of the funding period.
The principle aim for WP7 was to facilitate project awareness and the information sharing through the project Partners, scientific community, and the wider public. This information sharing was primarily
achieved through the creation and maintenance of the TB PAN NET project website and e-platform for communication among project Partners. Project information was disseminated to the scientific community via the ERS and the public via the ELF using the website, conferences, workshops, seminars and journals.

Project Results:
The following pages, the list of main achievements of the project is reported with a brief description of main activities. Further details are reported in the referenced deliverable documents. Deliverables and milestone description are also reported.

Work Package 1 - Molecular epidemiology of drug resistant strains in selected metropolitan settings in Western and Eastern Europe
WP1 LEADER: AO S. GERARDO

Partner involved: P1 USR; P2 HPA-MRU; P3 FZB; P5 AO S. GERARDO; P8 SMI; P13 LIC; P15 TUH; P16 VULSK; P17 VU IBT; P23 IPL ; P25 IPH ; P28 REUH ; P29 FCSR

Results Achieved

D1.1: International integrated clinical and molecular database for the control of the emerging problem of drug resistant TB spread in the metropolitan areas. We correlate molecular epidemiology and clinical data, identify national and crossborder TB outbreaks, we evaluate the relationship between the emergence of particular genotypic pattern to clinical outcome and multi-drug resistant TB.

A database for the centralized data collection was released in late 2010. Access to the database was restricted to WP1 partners and each partner could access and modify only his own data using a personal username and password. Patient data collected included epidemiological information (such as age, gender, country of birth, country and city of residence with ZIP code) and risk factors (living in a long term facility, homelessness, detention, alcohol and drug abuse, HIV status, immunosuppressive therapy). Information regarding clinical presentation of TB episode and strains details were also collected. MIRU-VNTR and spoligotyping analyses were manually imputed or imported by "copy and paste". Batch importation of data was also supported by the data base. Data collection included drug susceptibility for first line drugs results (with the possibility of adding results for selected second line drugs) and, when available, results of molecular tests for resistance to isoniazid and rifampin. The database was used by partners to collect the data relevant for the WP1.

D1.2: Report on molecular surveillance of MDR-TB spreading in metropolitan European cities related to the different socio-economical setting and to the ethnicity of the resident immigrant population.

Due to initial delays in data collection, a provisional report was released on M18 and the final report was released on M30.

By the time of the report release, a total of 2404 records with drug sensitivity test (DST) available had been entered. Clinical and molecular data were collected on 1132 MDR-TB cases with DST
been entered. Subsequent data collection brought to a final dataset of 6305 patients with available DST. Complete epidemiological and risk factors information were available for Tartu, Vlinius and Riga, while such information were available for a subset of the patients diagnosed in Milan and Bruxelles. Other Partners were able to provide information regarding age, gender and country origin of the patient, whilst no information regarding risk factors for TB acquisition were available. Multi-drug resistant strains included in the report were 164 (6.8%). The definitive database now includes 568 (9%) patients with MDR-TB. Patients with MDR-TB appeared to be more likely to be local-born, male, alcohol abusers and homeless, to live in a long-term care facility or to have been in prison during the previous 2 years. Differences between MDR and not-MDR TB patients were mostly driven by the higher prevalence of MDR-TB in Baltic cities, where the epidemic was mainly driven by local born patients, in whom traditional risk factor for TB, such as alcohol or drug abuse, recent detention and homelessness, were more prevalent than elsewhere. However, when regional differences were taken into account, being born in a foreign country was marginally associated with MDR-TB, whilst alcohol abuse was associated with MDR-TB risk in Vilnius and, marginally, in Western cities, but not in Tartu. There was no clear association between HIV-status and MDR-TB, even after accounting for regional differences.

D1.3: Report on the identification and comparison of the different risk factors and of the chains of transmission, suspected and unsuspected, in the different contexts of the European metropolitan areas

Due to initial delays in data collection, the report was released on M36. By the time of the report release, a total of 2225 collected strains were typed with 24-loci MIRU-VNTR typing. Current database includes 6008 full MIRU-VNTR 24-loci genotypic profiles. Clusterization rate, stratified by centre, ranged from roughly 20-25% in centres located in Western Europe to 40 to 60% in those located in Baltic countries. As compared with those harbouring a strain with a unique pattern, patients infected by TB strains sharing the same 24-loci MIRU-VNTR pattern were not different in terms of age, whilst they were more likely to be male and local-born. However, when the analysis was stratified by centre, no difference in terms of country of origin was found between clustered and non-clustered patients in Baltic centres, whereas local-born patients diagnosed with TB in cities located in Western Europe were confirmed to run an higher risk of TB clusterization. With regard to factors associated with TB transmission, institutional residence, recent detention intravenous drug abuse and alcohol abuse were more common among patients involved in clusters than among those who were not. At the time of the report, there was no clear association between HIV-status and risk of clusterization. However, in subsequent analysis HIV-positivity was associated with a higher risk of clusterization as compared with patients with a negative test, although the low number of patients for whom results of HIV testing were available prevented from definitive conclusions. The risk of clusterization appeared to be higher among patients diagnosed with MDR-TB than among those with susceptible or other form of resistant TB.

D1.4: Collaborative network among European Capital cities to define the shared main issues besides the assessment of the particular settings related to the different geographical, cultural and economic
Standards in genotypic data collection, reporting and interpretation were agreed and adopted by laboratories serving 8 metropolitan settings in Europe (London, Hamburg, Milan, Brussels, Lille, Tartu, Vilnius, Riga). Laboratories performing genotype analysis for strains isolated in these metropolitan areas attended intensive technical trainings and underwent strict external quality assessment. This ensured an appropriate implementation and use of 24-loci MIRU-VNTR typing or collaboration with the reference laboratory of Lille for genotyping archived strains.

Similarly, common standards for clinical data collection were agreed. This focused the attention of the network members on categories at risk for TB clusterization, thus allowing integration between clinical and genotypic data and detection of suspected and unsuspected chains of transmission between groups at risk.

D1.5: Definition of new surveillance strategies, based on a more systematic identification of the general and specific matters, updated to the actual epidemiological and social changes involving European countries

MIRU-VNTR typing using 24 loci was defined as the common method for M. tuberculosis surveillance within European Metropolitan settings. Analysis of hypervariable loci integrates 24-loci MIRU-VNTR for Beijing strains cluster definition.

Integration of clinical and epidemiological information with updated genotypic information allowed detection of TB strain circulation beyond country borders.

5 Major cross-border clusters were identified. Three of them included Beijing strains, involving 250, 120 and 33 isolates each, with a proportion of MDR isolates ranging from >80% to 30%. Other cross-borders clusters were caused by LAM lineage, with a far lower proportion of MDR isolates.

Study of traditional risk factor for TB transmission allowed to identify a significant prevalence of recent imprisonment among patients involved in one of these cluster, suggesting that former prisoner may have been the route of dissemination of this strains within Europe.

D1.6: Addressing the earlier diagnostic strategies and the control programs towards high-risk groups to decrease TB incidence not only into risk groups but also into the general population

Integration of genotypic and clinical data allowed identifying possible ongoing transmission within group at risk. Homelessness, recent detention, intravenous drug abuse and alcohol abuse were found to be associated with a higher risk of clustering. Also, HIV-positivity was associated with a higher risk of clustering as compared with patients with a negative test, although the low number of patients for whom results of HIV testing were available prevented from definitive conclusions. These groups of patients at risk of infection merit focused interventions in order to reduce incidence of TB and reduce the risk of TB dissemination for the general population.

As for MDR-TB, MDR-TB clustering was more likely to occur among homeless. Moreover, a trend towards a higher risk of MDR clustering, although not statistically significant, was found among HIV-infected patients.
D1.7: Integration between the genotypic pattern data and the researches concerning pathogenesis and virulence of MDR and XDR M. tuberculosis

Local and global cluster analysis allowed identifying MDR-TB strains capable of large spatial diffusion, as a proxy of their virulence. Hypervariable loci typing and whole genome sequencing are being investigated, as means to improve discriminating power of MIRU-VNTR, esp. for Beijing strains. Characterization of macrophagic response to different strains and lineages, characterized by different virulences, and identification of smallRNAs as possible markers of strain virulence were studied in WP2 and still are undergoing.

D1.8: Development of a multifunctional web based database that, by combining automated strain identification with clinical and geographic information, will represent a completely new tool for local and global TB surveillance

EpiScanGISplus, developed through the effective cooperation between Croatian National Institute of Public Health, Zagreb (Croatia, MD Vera Katalinic-Jankovic), Department of Periodontology, Münster (Germany, Prof. Dr. Dag Harmsen) and Research Center Borstel, Borstel (Germany, Prof. Dr. Stefan Niemann) since 2010, and used as comprehensive TB surveillance system for Croatia, was applied to the TB-PANNET dataset. Besides manual analysis, features like strain assignments by similarity search and tree-based algorithms for phylogenetic and minimum spanning tree reconstruction, EpiScanGISplus stands out by a bunch of automatically performed background processes during import such as nomenclature assignment to MLVA MtbC15 patterns as well as reporting of new MLVA MtbC15 patterns to MIRU-VNTRplus nomenclature server for type assignment, drug susceptibility identification (if a strain’s drug susceptibility characteristic is sensitive, resistant, MDR or XDR), strain clustering based on MLVA MtbC15 pattern, mapping as well as spatio-temporal analysis of TB cases and clusters. EpiScanGISplus is integrated with the strain typing system MLVAplus, the Geographical Information System (GIS), as well as with spatio-temporal scan statistics software SaTScanTM. Considering statistics feature, through its extension, ratios of male to female by age group, percentage of male and female by age group, and clustering rate are calculated for integrated TB cases in EpiScanGISplus. SaTScanTM software was integrated into EpiScanGISplus properly and adjusted to allow for automated prospective spatio-temporal cluster detections. Spatio-temporal clusters of special interest can be chosen to be visualized on map, where they can be investigated in geographical context, for example, considering the background of regional characteristics as terrain, city areas, infrastructures in form of highways and streets, cities, population density or incidences for the current period.

D1.9: Where necessary, Partners were trained for MIRU-VNTR typing, and for molecular epidemiological and phylogenetic interpretation of typing data

In order to ensure an appropriate implementation and use of the method among the WP1 partners, two 3-day intensive, hands-on technical trainings on MIRU-VNTR typing were organized, including calibration, validation, special case interpretation and troubleshooting. Finally, a second-level training, focused on epidemiological and genetic interpretation of typing data, was organized, involving all participants to WP1. The topics discussed included definition of clusters (e.g.
treatment of MIRU-VNTR single-locus variants, double alleles, combination or not with spoligotyping), phylogenetic predictions, use of available informatics tools (MIRU-VNTRPlus) and a perspective on upcoming genome-based developments. The training took place
Following these trainings, standards in genotypic data collection, reporting and interpretation were agreed and adopted by laboratories serving 8 metropolitan settings in Europe participating to the WP. This ensured an appropriate implementation and use of 24-loci MIRU-VNTR typing in all centres. When immediate implementation of the technique was not possible, collaboration between centres with different means and technical capacities allowed genotyping of archived strains by the reference laboratory of Lille.

D1.10: Quality assurance and support for typing performed for 1800 M. tuberculosis isolates collected within the project

To control and ensure consistent, high-quality 24-locus MIRU-VNTR typing among all the partners, technical trainings were organized in Lille. Proficiency testing, involving centralized re-typing of a subset of strains already typed by collaborating centres, was performed to test reproducibility of the results. Focused troubleshooting and (re)-training was organized on-site for centres with low performances at proficiency testing. Another, 2-day training was organized in Lille to ensure consistent molecular epidemiological and phylogenetic data analysis and interpretation.
In parallel, the reference laboratory of Lille (IPL) provided assistance for 24-MIRU-VNTR typing of isolates from Estonia and Latvia.
In addition, IPL performed subtyping by 4 hypervariable MIRU-VNTR loci of 650 isolates from different partners corresponding to the 4 major 24-locus-based clusters (two Beijing and two LAM) identified in the total TB-PAN-NET dataset, complemented by whole genome sequencing of 122 isolates belonging to the two largest Beijing clusters. Analyses of the results are ongoing.

Milestone N. Milestone name Due date Comments
M1 Availability of an international integrated clinical and molecular database for the control of drug-resistant TB spread suitable to correlate molecular epidemiological and clinical data, able to focus on phenomena into the TB outbreaks M6 Software available
M2 Agreement on protocol for molecular surveillance based on collected data and establishment a collaborative network among European Capital cities to define the shared issues besides the assessment of the particular settings related to the different geographical, cultural, and economic background of each metropolis M36 Document available
M4 Availability of final material for training on automated MIRU-VNTR typing M26 Training material available

Work Package 2 - Characterization of novel mutations involved in drug-resistant phenotype and virulence markers
WP2 LEADER: USR

Partners involved: P1 USR; P2 HPA-MRU; P3 FZB; P7 FIND; P8 SMI; P11 UNISI ; P12 Hain GmbH; P17 VU IBT; P25 IPH ; P27 UMDNJ ; P29 FCSR

Results Achieved
D2.1: Generation of strains phenotypically resistant to selected drugs

Strains of M. tuberculosis, all susceptible for 1st and 2nd line drugs, with different genetic background have been selected and used in the resistant mutant selection process to Amikacin (AMK), Ofloxacin (OFX), and Capreomycin (CAP). After selection, all mutants were retested by conventional culture/DST methods to confirm DR. Altogether 21, 30, 50 and 24 mutants selected on AMK, OFX, CAP and KAN containing plates, respectively, were generated. A subpopulation of the mutants was further analyzed by sequencing to detect resistance related mutations in gyrA, rrs and tlyA. Also the level of resistance (MIC) and possible cross-resistance were examined and the MIC was evaluated. As expected a certain degree of cross-resistance was seen among the aminoglycosides.

A set of monoresistant M. tuberculosis strains was generated considering also isoniazid (INH), rifampin (RMP), and para-aminosalicylic acid (PAS). Whole genome analysis (Illumina) on an EMB resistant variant without mutations in common resistance genes was also performed. Overall, approx. 200 clones were obtained and stored in a reference strains library. This panel of strains is an essential tool for EQA development at European and worldwide level because will allow to perform EQAs for second line drugs using monoresistant strains.

D2.2a: Identification of novel mutations affecting genes and regulatory paths operating on drug metabolism
D2.2b: Identification of novel mutations responsible of DR in individualized anti-TB regimens and improved knowledge on the mechanisms of resistance
D2.3d: Description of novel mechanism of DR

Better understanding of the relationship relying between the genetic markers of DR and the clinical outcome will permit the establishment of new diagnostic tools with improved effectiveness.

We created large databases to collect data on the genetic variants observed in susceptible and resistant clinical isolates. Mutations occurring in genes encoding putative targets for anti-TB drugs detected by sequencing have been included together with phenotypic and genotyping data to evaluate the correlation between the polymorphisms detected, the DR phenotype observed and the genetic background. Minimum Inhibitory Concentration and enzymatic activity were considered to solve specific phenotype-genotype correlations.

We focused on pyrazinamide (PZA, approx. 2000 isolates), fluoroquinolones (FQ) and second-line injectable drugs (SLID, 508 isolates). Concerning the pncA gene leading to PZA-R, we detected four classes of genetic variants: 1) very high confidence resistance mutations: only found in PZA-R strains, 2) high confidence resistance mutation: found in more than 70% of PZA-R strains, 3) mutations with an unclear role: found in less than 70% in PZA-R strains and 4) mutations not involved in phenotypic resistance. Despite a clear hot-spot region could not be found, the most frequently affected regions (representing more than 70% of mutated cases) were found at the promoter (-13 to -3), and at codons 6-15, 50-70, 90-100, 130-145, 170-175. (Miotto et al. MBIO 2014).

Targeting the gyrA and gyrB genes 81% of FQ-R cases were detected, with D94G and A90V as the most common mutations in the two collections considered (VU-IBT and USR/FCSR); considering rrs, eis and tlyA genes around 86% of SLID-R cases were identified, with g-10a and a1401g as the most common mutations found in the VU-IBT and FCSR/USR collections, respectively.

The inclusion of gyrB for the detection of fluoroquinolone resistance and of eis-tlyA for second-line injectables in new molecular tests could allow to increase the diagnostic performances, depending also on the local patterns of resistance.
the genetic background. These data were found important to update the Genotype MTBDRsl assay to its version 2. Analysis of other genes putatively involved in FQ-R such as Rv1634, carD, Rv3361c-Rv3365c operon, Rv3366, Rv3365c, Rv3364c, Rv3363c, Rv2688c allowed to identify SNPs not associated to phenotypic resistance. Analysis of other genes putatively involved in AG-R such as aac1, Rv1010 (ksgA), Rv1644 (tsnR), Rv3919c (gidB), transcription factor Rv1397A (whiB7), Rv0262c (aac(2')-lc), Rv0428c, Rv0730, Rv802c, Rv0919, Rv2170, Rv2775, Rv2851c, Rv2867c, Rv3027c, Rv3225c allowed to identify SNPs not associated to phenotypic resistance.

Increasing body of evidence reports discrepancies between molecular tests and MGIT DST for rifampicin (RIF) resistance. We correlated rpoB gene mutations (ranging from codon 511 to 535), MGIT DST, and MIC determination on agar medium to address these discrepancies. We selected 186 clinical isolates, including strains showing an rpoB NO WT + NO MUT hybridization patterns on WHO recommended LiPAs and control isolates with well-known mutations e.g. 531L) or wild-type for the rpoB gene. Sequencing data were analysed for the rpoB (hot-spot + N-term regions), rpoA and rpoC genes. all the mutations considered were associated to RIF-R on 7H10. Interestingly, mutations associated to low/medium MIC values were also associated to a delay in growth rate on MGIT (D516Y, H526 (N/C/S), L533P). There is a significant difference between MGIT delays in MGIT S/7H10 R vs MGIT R/7H10 R and also between MGIT delays in MGIT S/7H10 R vs MGIT S/7H10 S. Using an in silico model to analyse the impact of amino acidic substitution on the structure of RpoB, we found that all the mutations considered cause loss of RpoB-RIF interactions. Analysis of rpoAC genes showed putative compensatory mutations were found in 44 isolates. We also investigated the role of mutations occurring in genes encoding putative target for other drugs used in anti-TB regimen. The second-line antibiotic p-aminosalicylic acid PAS targeting the dihydrofolate reductase (DHFR) after activation by the dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS), enzymes of the folate pathway. This study described the implication of the enzyme (RibD), a putative functional analogue of DHFR. Indeed, the analysis of RibD in the collection of strains confirmed the existence of PAS resistant strains encoding a RibD over-expression mutations.

These studies provide a first insight on the European SNPs-genotype distribution in resistant clinical isolates. Large public data sets constitute the basis for new understanding of molecular patterns associated with DR mechanisms and are precious tools for the design of novel molecular assays.

D2.3a: Publicly available large-scale M. tuberculosis transcription-regulatory network
D2.3b: Website on M. tuberculosis gene regulation

Antisense regulation of genes relevant for the pathogenesis of M. tuberculosis:
The study of intracellular growth rate of resistant strains and the evaluation of intra-phagosome gene expression profiles provided information about the correlation among mutations responsible of DR phenotype and virulence features. After identifying sRNAs in M. tuberculosis, a computational analysis showed that AS sRNAs preferentially regulated transcription of membrane-bound proteins. Genes putatively regulated by novel cis-encoded sRNAs were enriched for two-component systems and for functional pathways involved in hydrogen transport on the membrane (Pellin et al. PLoS ONE 2012; Miotto

PLoS ONE 2012). A list of genes relevant for the pathogenesis of TB has been matched with AS sRNAs identified within this project. Overall, integrating 832 genes selected by direct literature search and 2276 genes found by the use of TuberculList, a complete list of 2358 genes putatively relevant for the pathogenesis of TB were identified. These coding regions were matched with 416 AS sRNAs identified in Mtb(P lli t l PL SONE2012 Mi tt PL SONE2012) A l f284AS RNA f dt b
Mtb (Pellin et al. PLoS ONE 2012; Miotto PLoS ONE 2012). A pool of 284 AS sRNAs were found to be cis-encoded to relevant genes for the pathogenic features of Mtb.

The sigma factor network of M. tuberculosis:
In this work, we have reconstructed the sigma factor network using two-plasmid method, an experimental strategy that tests direct, inter-sigma factor interactions in E. coli and validated the results in M. tuberculosis.
Reconstruction of network identified direct transcriptional interactions among all the sigma factors. Further, it will help to generate hypotheses concerning the effects of manipulating particular regulatory nodes on the survival of tubercle bacilli under stress conditions in culture and in infected animals.
We performed similar experiments with all the alternative sigma factor promoters. SigA, SigE and SigH transcribes sigE
Together, these results showed that (i) sigE is autoregulated and (ii) the autoregulation is mediated through promoter P2. We also validated the targets of SigB found in E. coli assay in M. tuberculosis by tetracycline-inducible system (data not shown). The entire network of M. tuberculosis sigma factors is depicted.
A Website on M. tuberculosis gene regulation has been developed:
http://ibl.mdanderson.org/~dfveiga/Tuberculosis/david.html

D2.3: List of sRNAs identified in MTB

List of sRNAs identified is available on Miotto et al. PLoS ONE 2012; Pellin et al. PLoS ONE 2012 and on the annual report

D2.3c: Database of network response in various growth arresting conditions for MDR/DR versus pan-sensitive strains

We developed an algorithm for predicting targets for the trans-encoded sRNAs discovered in Mtb. To gain insight in the function of the newly predicted sRNA-mRNA interactions, we analysed GO term enrichment and INTERPRO protein families within sRNA regulons by the means of a Fisher’s Exact Test (FET). Out of 96 subnetworks, 18 could be linked to biological processes (p-value of 0.01).
We performed the characterization of the expression of selected sRNAs in strains belonging to different lineages and under different growing conditions. We compared in a proof-of-principle study, M. tuberculosis Beijing strains with the reference strain M. tuberculosis H37Rv NCTC. The Beijing genotype is classified as modern lineage and have been reported that modern strains cause lower pro-inflammatory response and correlate with higher rate of progression to active disease, with higher bacterial load and drug resistance emergence. Our results suggest that some sRNAs are differentially expressed in Beijing strains during aerobic growth/hypoxic stress. sRNA expression profile has been also performed during antibiotic stress response, and analysed with mRNAs expression profiling.

D2.4: Identification of SNPs conferring a significant level of resistance by functional validation in mutant strains

The generation of isogenic mutants for SNPs considered to be involved in DR phenotype and the functional validation of the D2.4 gene(s) in the wild type M. tuberculosis H37Rv NCTC.
comparison with parental strains for DST will confirm the contribution of identified mutation. For plating experiments, H37Rv as well as a one susceptible Beijing strain have been selected. The panel for antibiotics applied for in vitro selection was extended to isoniazid (INH), rifampin (RIF), ofloxacin (OFX), moxifloxacin (MOX), levofloxacin (LEV), capreomycin (CAP), amikacin (AMK), kanamycin (KAN), and para-aminosalicylic acid (PAS). Resistant clones were checked by re-growing them on the antibiotic concentration used for selection, chromosomal DNA was isolated and resistance genes were sequenced. Overall, 250 clones have been stored in a reference strains library.
P1 USR/P29 FCSR and P25 IPH shared a database of 52 MDR clinical isolates phenotypically resistant to ethionamide, ETH. Complete sequencing data were collected for ethA (encoding the mono oxygenase EthA activating the drug), ethR (encoding the transcriptional regulator of EthA), ethA-R intergenic region, inhA (promoter and coding regions, encoding the target of ETH, shared with INH), mshA (encoding a glycosyltransferase), and ndh (encoding a NADH dehydrogenase) genes. Phenotypic DST to rifampicin and isoniazid, as well as rpoB, katG, inhA genes analysis are also available. Spoligotyping was performed identifying among others the 40.4% of strains belonging to Beijing family.
The validation of novel mutations detected in clinical isolates in ethA and ethR genes have been performed by cloning approach. Copy of the ethA gene, including the corresponding promoter region and the regulatory gene ethR was cloned into the integrative vectors pRP36 pRP37 in two opposite directions. The empty cloning plasmid pYUB413 was also used as a control.
A total of 25 strains was complemented (including H37Rv and 8 strains harbouring a wt ethA). Visual MABA was used as screening tool to compare the MICs for the transformed and the parental not transformed strains. As additional control ethA-R region was sequenced after plasmids integration to verify the restoration of the wild type pattern.
The developed model allowed to identify the novel mutations Tyr147Stop, Trp289Stop, Thr314Ile, Phe282Val in ethA gene and not associated with inhA mutations responsible of the emergence of ETH-R or at least to increase the ETH MIC. Similarly, frameshift insertion at codon 151 and deletion at codon 37 with inhA wt seem to play a role in the emergence of phenotypic resistance. No reduction of MIC was observed for strains harbouring frameshift insertions at codon 247 and 291, mutations Arg239Gly and His281Pro in the ethA gene: however the same strains showed concurrently a mutated inhA that could explain the resistance to ETH.
Four strains harbouring wt ethA and inhA showed anyway a MIC reduction after transformation compared to parental strains, thus suggesting a dose-dependent machinery relying on a regulating mechanism not associated with SNP in the ethA-R (results merged with D 2.2a – 2.2b -2.3d).

D2.5: Reliable assays to perform molecular DST on SLD and important anti-TB drugs
Development of GenoType®MTBDRsl version 2.0.
Based on the data provided by the Obj 2, a new generation of LiPA assay for the rapid detection of mutation in eis gene (GenoType®MTBDRsl version 2.0) has been developed. The diagnostic validation of the GenoType MTBDRsl 2.0 was performed by the following TB PAN NET partners: M. Cirillo (Emerging Bacterial Pathogens Unit, Milan), S. Rüsch-Gerdes (National Reference Center for Mycobacteria, Borstel), and S. Hoffner(Swedish Institute for Communicable Disease Control, Solna). Briefly, compared to culture/DST, the GenoType MTBDRsl 2.0 showed a few discrepancies to the method of comparison (culture/DST) at each study site. With respect to the fluoroquinolones the discrepancies at the study sites resulted in diagnostic sensitivities between 67,7% and 93,9%, diagnostic specificities between 98,5 % and 100 %, positive predictive values (PPV) between 96,9 % and 100 % and negative predictive values (NPV) between 84,7% to 97,1%.
(NPV) between 84.7% and 97.1%. With respect to SLID resistance compared to culture/DST the following performance data were achieved: diagnostic sensitivities ranging from 86% to 87%, diagnostic specificities ranging from 22.2% to 93.2%, PPVs ranging from 86% to 91.4% and NPV ranging from 14.3% to 90.8%.

Taken together, the data demonstrate considerable discrepancies of the GenoType MTBDRsl 2.0 results for SLID resistance compared to culture/DST. In contrast, the discrepancies of the GenoType MTBDRsl 2.0 test results regarding fluoroquinolones resistance testing are in an acceptable range, but for the diagnostic sensitivity and the NPV, the performance data are even close to 100%.

Besides the comparison of GenoType MTBDRsl 2.0 results to culture and DST results, the discrepant test results were also compared to the sequence data. Comparison of the GenoType MTBDRsl 2.0 results to sequence data resulted in a diagnostic sensitivity, diagnostic specificity, PPVs and NPVs of almost 100%. The data described based on current test results. However, at study site one and two, there are still some discrepant samples, which needs to be sequenced for further clarification (No. 5517, BTB-04-370, XTB-09-021, XTB-11-075). If the sequence data of these samples are in accordance to the GenoType MTBDRsl 2.0 test results, the performance data of the MTBDRsl 2.0 compared to culture/DST and sequence analysis as the reference method will even be better.

Taken together, the current results of the validation of the GenoType MTBDRsl 2.0 with clinical culture samples demonstrate a very high accordance (almost 100%) of the GenoType MTBDRsl 2.0 to the sequence data. The data clearly demonstrate, that phase 1 of the diagnostic validation of the GenoType MTBDRsl 2.0 (culture samples) was successful. Therefore, as a next step phase 2 (diagnostic validation with patient specimen) was started. Thus, appropriate study plans for phase 2 were currently prepared. The studies are ongoing.

Development of a test system for the molecular genetic detection of resistance to second-line drugs in M. tuberculosis

Following the knowledge of the molecular mechanisms leading to fluoroquinolone (FQ) and second-line injectable drugs (SLID) improved during the project, P1-USR/P29-FCSR developed a lab-on-chip assay (Veredus-STMicroelectronics) targeting gyrA (FQ) and rrs, eis (SLID) genes. An array layout (400 spots) was designed including specific probes complementary to the positions affected by the most common mutations in the three genes. Optimization was performed on clinical isolates showing different molecular patterns. A preliminary layout of probes that work with good sensitivity and specificity on clinical isolates till a concentration of 100 mycobacterial genome copies/μL was obtained. The selected probes allowed to cover all the positions of interest for gyrA, rrs and eis genes.

Development of a test system for the molecular genetic detection of pyrazinamide (PZA) resistance in M. tuberculosis

WP2 partners submitted in August 2014 a paper describing the role of mutations affecting the pncA gene in the emergence of phenotypic resistance to pyrazinamide (PZA) (Miotto et al. MBIO 2014). As reported, pncA gene requires whole gene analysis to detect resistance.

P1USR/P29 FCSR developed a lab-on-chip (Veredus-STMicroelectronics) for detection of PZA resistance. A first layout (126 spots) of a microarray including probes complementary to all the pncA wt gene sequence was released by the manufacturer. DNAs extracted from H37Rv strain (expected wt) and clinical strains carrying mutations scattered in pncA gene were evaluated. Master Mix composition and hybridization conditions were modified after a first screening and an alternative approach labelling (cy5)
FW primer together with labelled HV primer, designing probes complementary to the two strands, has been considered.

D 2.6: Suitable “home-made” and commercial RNA assays to detect bacterial viability and/or disease status

Mycobacterial RNA viability assay

A mycobacterial RNA-based viability assay based on detection and quantification of specific mRNAs in sputum, including icl, rrnA-P1, hspX, and fbpB was developed and extensively validated in London and Samara. This included development and validation of the methodologies for a total RNA isolation from sputum, its clean up and quantification, as well as primer and/or probe re-design for the qPCR, modifications to the procedure of the RT-PCR controls preparation (serial dilutions of cloned pAW109 RNA with known concentrations/copy numbers).

Results of initial validation experiments with the mycobacterial RNA specimens isolated from the cultured Mycobacteria and serial dilutions of standard mycobacterial DNA demonstrated good qPCR efficiency indicating optimal design of primers and probes (see 2013 Annual Report). However, results of qPCR were unsatisfactory with a very high Ct values for the NRTC suggesting high levels of DNA contamination. Therefore, we have implemented changes to the RNA isolation procedures and, specifically, tested a number of protocols for the reverse transcription to identify problems and improve results.

Extensive optimization allowed to identify optimal combination of the RNA isolation and cleanup (multiple treatment with the DNAfree Turbo (Invitrogen, UK) followed by the reverse transcription using either Quantitect (Qiagen, UK) or iScript (BioRad, UK) kits). This allowed to remove traces of the DNA, improve the performance significantly, especially with the fbpB and hspX genes and achieve >10 cycles difference between PC and NRTC indicating good performance and absence of the DNA contamination.

Conclusions:
1. Protocol for Mycobacterial RNA extraction, cleanup and quantification has been developed and optimized
2. Optimal conditions and kits for reverse transcription and cDNA generation have been identified

Serum miRNA

We aimed to identify a serum miRNA signature to be used for the diagnosis of tuberculosis (TB). To account for variations due to the genetic makeup, we enrolled adults from two study settings in Europe and Africa. The following categories of subjects were considered: healthy (H), active pulmonary TB (PTB), active pulmonary TB, HIV co-infected (PTB/HIV), latent TB infection (LTBI), other pulmonary infections (OPI), and active extra-pulmonary TB (EPTB). Sera from 10 subjects of the same category were pooled and, after total RNA extraction, screened for miRNA levels by TaqMan low-density arrays. After identification of “relevant miRNAs” we refined the serum miRNA signature discriminating between H and PTB on individual subjects. Signatures were analyzed for their diagnostic performances using a multivariate logistic model and a Relevance Vector Machine (RVM) model. A leave one-out-cross-validation (LOOCV) approach was adopted for assessing how both models could perform in practice. The analysis on pooled specimens identified selected miRNAs as discriminatory for the categories analyzed. On individual serum samples, we showed that 15 miRNAs serve as signature for H and PTB categories with a diagnostic accuracy of 82% (CI 70.2–90.0) and 77% (CI 64.2–85.9) in a RVM and a logistic model, respectively. Overall, we identified a 15-miRNA signature that could be used for the diagnosis of tuberculosis.
classifification model, respectively. Considering the different ethnicity, by selecting the specific signature for the European group (10 miRNAs) the diagnostic accuracy increased up to 83% (CI 68.1–92.1) and 81% (65.0–90.3) respectively. The African-specific signature (12 miRNAs) increased the diagnostic accuracy up to 95% (CI 76.4–99.1) and 100% (83.9–100.0) respectively. Serum miRNA signatures represent an interesting source of biomarkers for TB disease with the potential to discriminate between PTB and LTBI, but also among the other categories. Our findings were published in (Miotto et al. PloS One 2013). The study has been also reported on World Biomedical Frontiers (ISSN: 2328-0166, http://biomedfrontiers.org/infection-2014-6-8/).

Using the data generated by this study, we established a set of targets (i) removing “not detectable” miRNAs and (ii) including miRNAs relevant to monitor anti-TB treatment side effects (e.g. liver, brain, kidney injury). We started the enrolment of TB patients under treatment follow-up. In parallel, we are evaluating miRNA signatures in LTBI and PTB individuals to better refine results obtained by pooled sera reported in (Miotto et al. PloS One 2013). Data analysis in progress

D2.7: Useful genetic markers specific for virulence and DR with clinical prognostic significance

Recent discrepancies between molecular DST and phenotypic DST raised attention to the well-known correlation between mutations in the rpoB gene and phenotypic resistance to RIF. Our project better characterized the relationship between specific mutations in the hot-spot region of rpoB gene and the level of drug resistance caused. This will help in better addressing clinical management of patients. Similarly, our project provided a clinically-useful classification of more than 200 mutations found in pncA gene, involved in PZA-R. Defining mutations involved in the development of drug resistance also allowed to identify mutations unrelated to drug resistance (see D 2.2a – 2.2b – 2.3d).

On the other side, to further understand the role of miRNA during the infection with Mtb strains belonging to lineages with very different virulence profiles: M. tuberculosis EAI 1797, Haarlem 2336 and Beijing 12594 were obtained from clinical samples cultured on Lowenstein/Jensen medium at the National Reference Center for Mycobacteria in Borstel, Germany.

miRNA profile by TaqMan Low density microRNA Array (TLDA) in human macrophages together with inflammatory cytokine production, intracellular mycobacterial survival growth, have been studied. Results showed that specific MTB lineages (ancient/modern) are able to provide different immune response during in vitro macrophage infection. These findings are relevant in better understanding the virulence of MTB.

Milestone N. Milestone name Due date Comments
M3 Generation of strains phenotypically resistant to selected drugs M24 Panel generated
M5 Computational determination of strain-to-strain differences in transcriptional response at the network level M36 Document available
M6 Development of molecular rapid- and easy-to-perform assays to detect mutations involved in DR M66 Assays available and tested

References
Work Package 3 - Development and standardization of EQA for drug-resistant TB and expansion of capability for culture-based and molecular-based techniques for drug-resistant TB

WP3 LEADER: FZB

Partners involved: P1 USR; P2 HPA-MRU; P3 FZB; P8 SMI; P9 SSI; P10 CNIPH; P13 LIC; P15 TUH; P16 VULSK; P18 NIPH Norway; P19 NRL Slovenia; P20 ITM; P21 SPF Brasov; P24 THL; P26 NRL Poland; P28 REUH; P29 FCSR

Results Achieved

D3.1: Data report from 1st EQA testing

Large multi-centre studies defining the accuracy of different pheno- and genotypic DST methods for 2nd line drugs are now available. The final results of the WP3 will help answering which methods can provide the clinicians with the most reliable results, necessary for an individualized treatment regimen. External Quality Control rounds have been performed in all laboratories for 1st and 2nd line drugs with a very high sensitivity and specificity. Nearly all laboratories involved had not only pass the EQA but had obtained a benefit for the routine performance of DST.

All partners have performed DST for 1st and 2nd line drugs and sent back the results to P3-FZB from the 1st round in 2009 and 2010. The final analyses have been done by P3-FZB in 2010. Unfortunately, the number of resistant strains for PAS and Cycloserine is too low for calculation of sensitivity and PPV. During the meeting in Borstel (P3-FZB) in 2010 participants have been asked to check their stock culture collection for resistant strains. Two laboratories have sent strains but after rechecking in the countries and in P3-FZB the number of PAS and Cycloserine was again too low to use them for an EQA panel. All other members have no resistant strains in their stock. During the WP3 meeting in Copenhagen (in Nov. 2011) all members agreed, that we will not include the two drugs in the EQA panel. Together with the strains, a questionnaire for description of the methods used has been created and sent back together with the results. P3-FZB has finalized the analysis in 2011 of the questionnaires for all drugs used. In 2014 all data have been published together with the results of the Baltic-Nordic TB Laboratory Network.

D3.2: Panel of isolates with well characterized mutations for 1st and 2nd line drugs are available

Well characterized strains with well characterized mutations and drug resistant patterns are available. Additionally, in vitro generated strains (D3.5) with mono-resistance are available for EQA.

D3.3: Data analysis, recommendations for detection of hetero resistance

Six strains, susceptible or monoresistant to rifampicin or isoniazid, were selected. The susceptible and
resistant strains were mixed at different proportions of resistant bacteria, from 100% susceptible to 100% resistant. Susceptibility tests with different methods on the selected strains were made after different, common pre-cultivation techniques. Heteroresistance was best detected with conventional phenotypic drug susceptibility test (DST), whereas the line probe assay and sequencing were not able to detect the clinically relevant 1% proportion of resistant bacteria. We also made experiments to evaluate the effect of different decontamination methods and the effect of storage time and temperature on the detection of heteroresistance. Our preliminary evaluation of the results indicates that storage and decontamination has little influence on the heteroresistance, which was best detected by DST, whereas molecular detection was less effective.

D3.4: Panel for EQA of the in vitro generated strains and molecular based DST with the extracted DNA in all countries.
D3.5a Data analysis from panel testing of the in vitro generated strains and molecular based DST with the extracted DNA in the core group

There is a pronounced need of scaling up proficiency testing to assure the quality of DST to second line drugs, and to the neglected first line drug PZA, for laboratories around the globe performing routine DST of these agents. At the same time it is preferable from a bio-safety perspective to avoid including widely drug resistant clinical isolates in proficiency test panels for EQA. To meet the increasing demand of EQA in a way related to less biohazards, sets of mono-resistant strains to each of the most important second line drugs used in the therapy of MDR-TB, second line injectables and fluoroquinolones were laboratory selected by picking spontaneous mutants related to a resistant phenotype for each of the drugs. Subsequently a similar set of laboratory selected PZA mono-resistant mutants was put together. When evaluated within our WP3 network, these selected mono-resistant strains were shown to give as good results, in both phenotypic based DST and molecular assay to detect resistant related mutations, as drug resistant clinical isolates. The mono-resistant strains selected and validated for use in EQA panels are kept for future use at the SRLs in Stockholm and Borstel.

D3.5.b: Phenotypic versus genotypic determination of RMP DR in Mycobacterium tuberculosis

Comparison of the level of phenotypic resistance detected for various rpoB mutants, showed full agreement between Löwenstein-Jensen (LJ) and MGIT 960 automated MGIT-DST for mutations located at codons 513 (Lys or Pro) and 531 (Leu, Trp), which were always resistant by both methods. For mutations 511Pro, 516Tyr, 533Pro, 572Phe, and several 526 mutations, LJ and MGIT results were highly discordant, with MGIT-DST failing to give a result or declaring the strains susceptible (Rigouts L, et al. J Clin Microbiol 2013). In parallel, a retrospective study aimed to document the prevalence and prognostic value of rpoB mutations with this unclear phenotypic resistance. The study design entailed sequencing directly from sputum of first failure or relapse patients without phenotypic selection and comparison of the standard retreatment regimen outcome, according to the mutation present. With 63% of patients experiencing failure or relapse in both groups, there was no difference in outcome of first-line retreatment between patients carrying a strain with disputed versus common mutations. We conclude that rifampin resistance that is difficult to detect by the gold standard, phenotypic DST, is clinically and epidemiologically highly relevant (Van Deun et al, 2013).

Analysis of routine phenotypic and genotypic DST data among 11 WP3 partners performing routine line-probe testing, revealed the absence of wild type (WT) bands without presence of specific mutation (MUT)
bands (ΔWT strains), at a rate of 7 to 28% among MDR-TB suspects. These isolates showed an overall good agreement phenotypic DST on solid medium, yet poor agreement in MGIT960 ranging from 33-87%. Part of these ΔWT strains were sequenced and we identified the same mutations at positions 511, 516, 526 and 533. Unfortunately, treatment outcome data are not available for all isolates/samples. However, the example from Croatia, lends support to the clinical significance of these mutants (manuscript in preparation).

D3.6: Data on EQA from 27 Countries

Three networks/projects involving 27 European countries were established to investigate the quality of second-line drug (SLD) susceptibility testing with conventional and molecular methods. 1) The “Baltic-Nordic TB-Laboratory Network” comprised 11 reference laboratories in the Baltic-Nordic States. They performed SLD testing in the first phase with a panel of 20 Mycobacterium tuberculosis strains. After several laboratories made technical changes a second panel of 10 strains with a higher proportion of resistant strains were tested. Although the concordance for Ofloxacin, Kanamycin, and Capreomycin was consistently high, the largest improvements in performance were achieved for the analysis of Ofloxacin resistant (from 88.9 to 95.0%), and Capreomycin resistant (from 71.0 to 88.9%) strains. 2) Within the FP7 TB PAN-NET project (EU Grant agreement 223681) a quality control panel to standardize the EQA (External Quality Assurance) for first-line drugs (FLD) and SLD testing for phenotypic and molecular methods was established. The strains were characterized by their robustness, unambiguous results when tested, and low proportion of secondary drug resistances. 3) The (European Reference Laboratory Network-TB) ERLN-TB network analyzed four different panels for drug resistance testing using phenotypic and molecular methods; in two rounds in 2010 the 31 participating laboratories began with 5 strains, followed by 10 strains and 6 additional crude DNA extracts in 2011 and 2012 were examined by conventional DST and molecular methods. Overall, we demonstrated the importance of developing inter-laboratory networks to establish quality assurance and improvement of SLD testing of M. tuberculosis.

D3.7: Report on Final WP3 meeting

14 members of W3 attend the last meeting in Borstel in 2014. As already discussed in former meetings Objective 1 (EQA) is finalized and published. It was agreed that no further action is necessary. From all rounds all clear resistant strains will be included in a strain collection. Only all WP3 members have access to the strains. For the objectives 2 and 3 Sweden and Germany reported the results, obtained in 2013/2014. Results of drug susceptibility testing performed with molecular based methods from inactivated and lab generated strains have been discussed. Regarding heteroresistance (Objective 4) Denmark showed the obtained findings in 2013/2014. For objective 5 (DST pheno- versus genotypic) Belgium reported the nearly finalized results. For all objectives the outcome has been deeply discussed with all participants. Final report summarising WP4 activities, main results and deliverables achieved was prepared and submitted to the EC as a separate document.

Milestone N. Milestone name Due date Comments
M7 Agreement on set of drugs for EQC M2 List of drugs approved by WP3 partners
M8R M8R M8R M8R M8R M8R M8R M8R M8R M8R
M8 Recommendations for use of a coded QC/EQA panel including both extracted DNA and suspended heat killed bacteria to be used for molecular demonstration of resistance conferring mutations in M. tuberculosis
M32 Recommendations delivered
M9 Recommendations on testing of hetero resistance borderline resistance for RMP M24
Recommendations approved
M10 Agreement of recommendations for SLD testing for European countries M64 Recommendations approved
M11 Availability of monoresistant strains for a “safer” EQA for SLDs, to be distributed in settings with low MDR incidence M20 Panel available for EQA
M12 Expansion to further countries, selection of candidates lab visits, discussion of methods used, training M36 List of selected Countries

References

Work Package 4- Development of integrated sites for clinical and diagnostic trials of new drug applications, new diagnostics, and development of markers for treatment success, and identify clinical and social factors, including HIV as markers of resistance
WP4 LEADER: HPA-MRU

Partners Involved: P1 USR; P2 HPA-MRU; P3 FZB; P7 FIND; P8 SMI; P12 Hain GmbH; P13 LIC ; P15 TUH; P16 VULSK ;P17 VU IBT; P21 SPF Brasov; P22 I-M. Nasta ;P28 REUH ;P29 FCSR

Results Achieved

D4.1: Baseline description and analysis of field sites: existing ethics process, governance, accounting procedure, TB/HIV rates at project field sites (from previous year to guide planning for objective 3). Collaborative research agreement signed based on Consortium Agreement.

Collaborative Research Agreements signed by P13/28, 15, 16, 22 and Samara (P2 sub-contractor) in September 2009.

Ethics review boards exist at each site, structure, composition and application costs at each site is known, application procedure, length of obtaining ethics permission and rules for applying to different (local, i.e. hospital-based or federal, i.e. ministry of health-based) levels are clarified for each site. Ethics approvals were obtained at all sites including QMUL (as a scientific leader of the studies) for the studies conducted with the PANNET project.

Preliminary situational analysis of each site was conducted in May 2009 using a structured questionnaire. Detailed information was collected on: epidemiology of TB, HIV and co-infection across the partner sites; assessment of existing clinical and laboratory infrastructures (human resources, equipment, beds)
necessary to assemble an MDR/XDR cohort; assessment of laboratory capacity to reliably perform microscopic, culture tests, (first and second line drug testing) FLD and SLD DST, molecular testing; safety levels; QA; work load; infection-control methods; existing ability to collect samples, logistics of sample transport to central site, and ability to export samples/cultures/DNA; financial accounting, legal aspects; current HIV testing policy and methods used, coverage of testing – regulations and real situation; preliminary identification of site-specific needs necessary to assemble and follow-up an MDR/XDRTB cohort to assess biomarkers and to conduct clinical trials of drugs and diagnostic equipment.

Extensive discussion of available resources, planned studies logistics, personnel, established diagnostic algorithms and introduction of novel tests took place during a meeting of trial sites research leaders in September 2009 in London. An action plan was developed and containing problems and weaknesses which needed to be addressed by each site regarding trials and cohort development. Within the following years the infrastructure at each site was significantly upgraded and currently all partners are able to act as a coherent network of centers capable of conducting TB-related research in different formats including diagnostic and clinical trials as well as epidemiological studies (see D 4.3 4.4 and 4.7). A number of successful studies was conducted within the PANNET project proving the sites’ capacities and ensuring sustainability of developed infrastructure including personnel training.

The rates of HIV-TB co-infection was established during the preliminary situational analysis: 6.5% (P13/28, Latvia); 10.3% and 0% HIV infection among TB and MDRTB patients (P15, Estonia); 0.4% (P16, Lithuania); 0.5% (P22, I.M.-Nasta); 16.9% (P2-Samara). Detailed analysis of the risk factors for MDR/XDRTB including the role of HIV as well as survival analysis of MDR/XDRTB patients was conducted within the planned studies (see D 4.4) after piloting the study questionnaire at the P2 Samara, Russia filed-site.

D4.2: Curriculum developed for common MDR-TB diagnosis training; course materials and training timetable for fellows include partners’ visits for training in London with the development of new materials and as well as WHO-approved modules.

Development of training curricula and providing training on clinical and diagnostic trials as well as development and validation of laboratory assays and quality control as well as clinical trial protocols was one of the key activities of the network capacity building and its infrastructure development. In Y1-2, training programs were developed and two courses organised for WP4 leaders, senior clinicians and laboratory coordinators from participating sites in September 2009 (Clinical and Diagnostic trials) and May 2010 (Laboratory aspects, QC and development of new biomarker assays) in collaboration with P1, P7, P12, P13/28, P15, P16. For laboratory workers, both theoretical and hands-on practical training was provided.

The following topics were covered:
Clinical and Diagnostic trials: Set-up: clinical governance, ethics, sponsorship, trial registration, protocol writing; Introduction into basic epidemiology and study design including choice of outcome measures, Phase 2 vs. Phase 3 clinical trials, general trial administration; Logistical issues: attaining recruitment targets, patient incentives; Introduction into a medical statistics including randomisation, blinding, unblinding, withdrawals, block stratification; Pharmacovigilance: monitoring for adverse events, adverse event reporting; Pharmacology: choice of intervention, pharmacokinetics; pharmacogenomics; Analysis: ITT vs. per protocol analyses; interaction analysis; reporting of study/trial results; Potential barriers to success of a trial.

L b t t QC dd l t f bi k N l b t i l MODS
Laboratory aspects, QC and development of new biomarker assays: Novel bacteriology assays: MODS and TLA; Main principles of conducting diagnostic trials, diagnostic tests development and pipelines; Biomedical statistics; Laboratory methods and principles of novel pathogen and host biomarker assays; Potential obstacles in conducting clinical and diagnostic research; Line-probe assays and other molecular methods; Molecular laboratory design, workflow and prevention of contamination; Quality control and quality assurance; design and analysis of translational studies.

Further training was provided through a series of site visits in Riga (P13/28), Vilnius (P16), Tartu (P15), London (P2) and Samara (Subcontractor) which ensured sustainability of changes and high quality of data generated.

D4.3: Assessment of strengths and needs for diagnostic and clinical trials at field sites and clinical field trials reported above with summary of demonstrated proficiency in microscopy and culture for field sites.

A detailed situational analysis looking at the existing laboratory and clinical infrastructure (available diagnostics resources, algorithms of routine diagnosis, personnel and laboratory workload) was conducted through a questionnaire-based survey and a series of visits to each of the trial sites by QMUL staff members aiming to further investigate existing and potential capacity of each site to take part in proposed research projects and to plan further training and procurement of necessary equipment/reagents. The situational analysis was followed by the development of a comprehensive action plan outlining laboratory needs and later - by implementation of a number of infrastructural improvements including a series of both laboratory and clinical trial training events (see D 4.2 and D 4.7). At several sites laboratories were optimised and expanded with a particular emphasis on molecular testing facilities including training of the personnel in all novel molecular techniques necessary to conduct the planned studies. A number of site visits to monitor the sites performance within the biomarkers study took place throughout the project. Logistics of specimen shipment was established and functions well. A system of distribution of laboratory work between the sites was set up and agreed. A system of archiving project material and creation of local biobanks was established and functions well across all sites. Quality control rounds of microscopy, DST and molecular tests have been completed, errors identified and rectified. EQA established by P2, P3 and P8 across main clinical sites.


Identified strengths and weaknesses of the sites were addressed through a series of trainings that led to improvements. To address the gaps in knowledge regarding the conduct of clinical, epidemiological and pharma trials and to further build sites’ capacity, Clinical Trials Training Courses were conducted at P2 in 2009 and 2010 for all field sites. Several staff members of P2 attended Good Clinical Practice trainings at QMUL in London. Multiple scientific staff exchanges occurred between P2 and partner sites in Lithuania, Latvia, Estonia and Romania as well as Samara (sub-contractor of P2), Russia and between Samara and these sites as well as other bilateral exchanges for protocol development, laboratory training and assay implementation exchanging best practice amongst field partners. A number of epidemiological and diagnostic accuracy studies were designed and conducted by the field sites and demonstrated the ability of the partners to act as a coherent network of study and trial sites. The
results were presented at a number of scientific events and were published (8 manuscripts) in peer-reviewed journals. More publications have been prepared for submission.

The rates of HIV among MDRTB patients and a more detailed analysis of the risk factors for MDRTB were assessed within the extended study protocol: “Retrospective analysis of multi- and extensive drug resistant tuberculosis (MDR/XDRTB) risk factors, survival and HIV co-infection rates: a multi-center study”. The study involved five sites and included a retrospective (risk factors case-control study) and prospective (MDR/XDRTB patients’ survival) components.

All patients with tuberculosis registered in 2009 at Lithuanian, Latvian (in addition patients registered for treatment in 2007 were recruited), Romanian (Bukharest only) and Estonian sites were recruited into analysis (2,045 patients including 749 MDR/XDRTB cases). This cohort is available for further prospective follow-up.

Men, young people, contacts of an MDR/XDRTB case were more likely to be infected with a resistant strain. MDR/XDRTB patients are more likely to have more severe symptoms of disease (chest pain, cough, weight loss). Urban living, unemployment and smoking increase the risk of acquiring MDR-/XDRTB during treatment. Patients with acquired MDR/XDRTB are less likely to have cavitatary disease. Treatment in the past especially with an unsuccessful outcome was strongly associated with the risk of MDR and XDR development during the following treatment cycles. Median survival time was 5.9 years both for MDR and XDRTB patients. Median survival time of HIV-infected individuals is almost 3 times shorter than survival of those non-infected. Older age, male gender, alcohol abuse, unemployment and lower levels of education were all independently associated with poorer survival. Later culture conversion was also shown to have an impact on survival. Prescription of SLDs significantly improved survival; the more SLDs were used the longer the patients survived.

The Lithuanian partner took part in the extended analysis of patients registered for treatment in 2002-2008 with P2 in addition to taking part in the multi-site analysis of patients registered in 2009, i.e. a significant expansion of the original objective. A 2002-2008 cohort included 10,664 TB patients (including 1,809 MDR/XDRTB cases) was analysed; the results were published as two scientific manuscripts in peer-reviewed journals. The study revealed that of 752 sensitive TB patients who underwent a second treatment cycle, 164 (21.8%) acquired MDR and 20 (2.7%) acquired XDR. Younger age, urban living, known TB contact, alcohol abuse and infection with Beijing family strains were independently associated with increased risk of primary MDR/XDRTB. Unemployment, smear-positivity at the second treatment cycle, and mono/poly-resistance were associated with increased risk of acquiring MDR/XDR during treatment. Median survival for MDRTB and XDRTB patients was 4.1 (95%CI 3.7 4.4) and 2.9 (95%CI 2.2 3.9) years. The difference in survival between MDRTB and XDRTB patients was not significant. Social factors, rural living, HIV-infection and Beijing bacterial strain family impacted on survival. Treatment with second-line drugs improved survival.

D4.5: Identification, development and performance assessment of RNA biomarker assays with additional complementary multiple cytokine/chemokine analyses as proof-of-cure assays for MDR-TB patients at multiple clinical sites.
D4.6: Development and preliminary assessment of performance in clinical trials

One of the key highly innovative activities within WP4 was a development of novel proof-of-cure biomarker assays which would potentially help to predict a treatment outcome, to determine whether a treatment regimen was well chosen and effective and establish the earliest point at which an individual was cured.
bacteriologically therefore improving infection control and reducing institutional cross-infection. Evaluation of novel biomarkers was done on two longitudinal cohorts of TB and MDRTB patients established and followed up for up to two years within the context of multicentre biomarker study conducted by four partners (P1, P2, Samara-subcontractor, P13/28, P15, P16) coordinated by P2 (London). Performance of new biomarkers was assessed against gold standard methods including smear microscopy, bacteriological culture and time to positivity on liquid media.

A comprehensive study protocol was developed jointly with the partner sites. Prospective recruitment of patients lasted 12 months (01/2011 – 01/2012) and follow-up was completed in March 2014. A total of 152 MDR TB and 156 non-MDR TB patients were recruited across three sites, followed up for <2 years and have their outcomes recorded (for all non-MDR and most MDR patients).

Within the project, a large biobank of human and mycobacterial specimens has been established. It contains samples collected prior to treatment initiation, samples collected during the treatment at multiple time-points, and samples taken at the end of therapy. All the specimens were collected in duplicate and triplicate which allowed us to create a centralised sample archive in London and also keep specimens locally at sites. The biobank contains over 27,000 specimens including plasma, serum, urine, blood, human DNA and RNA, M. tuberculosis cultures and DNA, sputum.

The length of the patients’ follow-up within the study as well as variety of the specimens collected and availability of detailed clinical/epidemiological/microbiological data make the biobank a unique source for further investigation. When signing the consent form, patients agreed for the specimens to be stored and used for a TB-related research beyond the project so these specimens are available for future research with a full compliance with all ethics aspects.

Within the project, four biomarker assays were developed and validated:

- Human mRNA gene expression assay (changes in expression of 18 TLR receptor and other genes involved in pathogen recognition) has been developed, validated and implemented at P2 (London and Samara). A total of 2381 human RNA specimens were collected across three sites in 2011-2014. Of these, 1824 were converted into the cDNA and analyzed using the TLR mRNA assays in Samara and London in 2011-2014. Data on relative gene expression was analysed in conjunction with the relevant clinical (outcomes) and laboratory (microscopy, bacteriological culture and days to positivity) data. Patients from three sites (P2, P13/28 and P16) were grouped into nine groups depending on their resistance profile and the speed of response to treatment.

Generally most genes were upregulated during the first 6 months which could be explained by an activation of the pathogen recognition pathways and immune system at initial stages of TB infection. Fast and slow responders can be distinguished using the TLR assay with fast responders demonstrating decline in many TLR genes (including TLR8 (transcript variant 2), MYD88 genes, TLR4, TLR6, TLR7) activity starting from Months 1 and 2. In slow responders downregulation of certain genes can be seen from month 5 only. In general expression in many genes followed similar patterns with levels for slow responders being higher and falling more slowly compared to fast responders.

Study on TLR expression genes can provide valuable information about patient’s response to TB treatment. As a result of this study, several TLR genes with changes in expression during the course of treatment have been identified. Based on results of our study, this assay has the potential to be considered and further evaluated as a proof-of-cure assay for MDRTB patients.

Mycobacterial RNA-based viability assay based on detection and quantification of specific mRNAs in sputum, including icl, rRNA-P1, hspX, and fbpB was developed and extensively validated in London and...
Samara. This included development and validation of the methodologies for a total RNA isolation from sputum, its cleanup and quantification, as well as primer and/or probe re-design for the qPCR, modifications to the procedure of the RT-PCR controls preparation. As a result of the study, protocol for Mycobacterial RNA extraction, cleanup and quantification has been developed and optimized. Optimal conditions and kits for reverse transcription and cDNA generation have been identified.

A Propidium monoazide (PMA) viability assay was developed in collaboration with P1 (Milan). Further development and validation of the assay on clinical specimens (sputum specimens liquefied using NALC) was performed within WP2 and WP4 by P2, P13/28, P16 using four different readout assays (Hain MTBDRplus v1; Hain MTBDRplus v.2; GeneXpert MTB/RIF G3 v.3 and GeneXpert MTB/RIF G4 v.5) and PMA final concentrations 100 uM and 500 uM on a total of 2434 liquified sputum specimens.

Sensitivity and specificity of the PMA assay compared to bacteriological culture tended to vary depending on the readout assay used. Using GeneXpert MTB/RIF, significantly better performance characteristics have been achieved with sensitivity being as high as 95.0% (Version G3) and 97.5% (Version 4) and PPV varying from 97.0% to 97.0%. Specificity was 80.0% (Version 3) and while using the version G4 it was lower varying between 52.8% and 70.7% depending on the final PMA concentration.

To address issues with assay specificity and adjust/optimise technical parameters of the readout assay for the reading and interpretation of the PMA results (specifically, cut-off Ct values on GeneXpert MTB/RIF assays for a test to be considered as positive or negative), a validation study was performed based on inclusion of the controls (specimens untreated with the PMA) into the study and comparing Ct values recorded for PMA-treated and untreated specimens. In the validation study delta Ct values in paired specimens collected at Months 1-2 and subsequent months of treatment (mean values 7.12 95% CI 3.52-10.72; and 7.97 95% CI 2.68-13.27) were significantly higher compared to those collected before the treatment commenced (0.71 95% CI -0.80-2.21) potentially indicating lower proportions of viable Mycobacteria in sputum specimens collected from patients at advanced stages of antituberculosis treatment. Spearman correlation analysis revealed a good correlation between Ct values and time to positivity ($r= 0.61; 95\%\ CI 0.54-0.67; p<0.0001$) on the MGIT microbiological culture system indicating the potential of the assay to detect bacterial load (as measured by days to positivity).

Levels of circulating pro-inflammatory cytokines and other molecules involved in cellular immune response pathways as well as their kinetics during TB infection were shown to be different in fast and slow responders indicating their potential role as biomarkers of treatment response and cure. In our study we analysed levels of thirty different soluble cytokines, chemokine and growth factors in serum/plasma samples taken from drug sensitive, non-MDR TB and MDR TB patients at different time points across multiple partner sites using an innovative Luminex platform (magnetic beads based multiplex ELISA assay).

A total of 1430 plasma specimens from three project sites were tested using the Luminex assay in London. In our analysis we looked at the levels on cytokines at time point 0 (before the commencement of treatment) and kinetics of cytokines and other analytes during anti-TB treatment. Levels of CXCL9 (MIG) and IL-6 were significantly higher in slow responders than those in fast responders ($p=0.0132$ and $p=0.0411$ respectively) while CXCL10 (IP10) level was significantly higher in fast responders ($p=0.0047$) suggesting that CXCL9 (MIG), CXCL10 (IP10) and IL-6 can be considered as biomarkers of treatment response. Differences in levels of other cytokines/chemokines between the two groups were not statistically significant.

To conclude, four laboratory assays (two pathogen viability assays and two host-related assays) have
been identified, optimized and extensively validated across the network. PMA viability assay, cytokine assay and TLR gene expression assay demonstrated their potential as biomarkers of disease, treatment response and cure.

Former D4.6 now D 4.7: Development and preliminary assessment of capacity for performance in clinical trials including enhanced protocol development for EBA, combination and higher dose drug studies.

The development of a Clinical Trial Network began with a detailed situational analysis of current partner capabilities and TB epidemiology. It allowed us to identify strengths and weaknesses of the sites and address deficiencies in infrastructure, laboratory performance and knowledge gaps through a series of both laboratory and clinical trial training events (see D 4.1-4.4).

The training and gained expertise allowed the partners to jointly develop and conduct a number of multi-center studies (studies on diagnostic accuracy of several EU SME commercial line-probe assays and a thin-layer agar color test, studies on identifying risk factors for drug resistance and retrospective and prospective survival of patients with MDR and XDRTB). All these diagnostic accuracy studies have involved development of complex study protocols, implementation of strict laboratory quality control and adherence to the standard operating procedures and agreed protocols. All the studies were successfully completed; a number of manuscripts were published in international peer-reviewed journals as part of our dissemination strategy and the rest of the data has been analysed, with draft manuscripts ready for submission.

Within a large multi-center study on novel biomarkers three participating sites (Samara, Latvia, Lithuania) performed prospective recruitment and follow-up of TB patients, regular sample collection and conventional laboratory testing, including microscopy, drug susceptibility testing and bacteriological culture (on solid and liquid media). The laboratory work is realigned across the network depending on available equipment, infrastructure and staff skills. Visiting scientists at different sites demonstrated excellent performance and ability of the laboratories involved in the WP4 to work as a concerted integrated network. A massive biobank of serial samples (plasma, serum, sputum, cultures, mycobacterial and human DNA, blood, human RNA, urine) was created centrally in London with duplicates being stored at each field site (see D 4.5-4.6).

Each field site is fully prepared from the clinical, laboratory and organizational aspects to conduct clinical trials in different formats. Estonian, Latvian and Lithuanian sites are now acting as field sites for the conduct of Phase 1 and 2 pharma trials of TB drugs (meeting the global capacity building aim of PANNET) and they proved to perform well according to international clinical trial standards. All sites have been actively involved in diagnostic trial studies. Numerous negotiations have taken place with other research trial groups involved in on-going clinical trials for MDRTB regarding possibilities of joining them with new sites. Legal, ethical, financial and other constraints were outlined for each site. The capacity to perform multiple different trial designs in addition to the original planned phase 1/11 trial was changed as new data was published which made the original trial on vitamin D redundant. Nevertheless a full vitamin D clinical protocol was developed, ethics permission sought, and presented for preliminary regulatory approval as planned. The London site worked to develop the protocol of the RIFASHORT trials (with partners from outside PANNET) with input from other PANNET partners on feasibility, ethics and health economics. Novel laboratory DST assays/methodologies and protocols were developed and initiated to support different trial designs.
Former D4.7 now D4.8: Final report including analysis of co-infection rates, DR factor analysis, proof-of-cure studies, training completed, and manuscripts prepared for publication.

Final report summarising WP4 activities, main results and deliverables achieved was prepared and submitted to the EC as a separate document (Deliverable 4.8 report).

Milestone N. Milestone name Due date Comments
M11 Assessment of needs for diagnostic and clinical trials at field sites and identification of resources to be committed M12 QC results, Proficiency demonstration
M12 Establishment of the equivalent laboratory infrastructure for research at all sites M24 Quality assessment results
M13 Enrolment of MDR-TB patients cohort (500 patients) M24 Completion of epidemiological data analysis
M14 Identification of the most appropriate proof-of-cure assay M48 Completion of analysis of Performance assessment

Work Package 5- Training on TB clinical management, control and research

WP5 LEADER: FSM

Partners Involved: P4 FSM; P6 ERS; P13 LIC

Results Achieved

D5.1: Task analysis on MDR-TB training

Preliminary task analysis of the target groups for the training course on MDR-TB management was performed in January and February 2009.
The target population for training was defined. The course was designed for individuals responsible for planning, organizing, implementing and evaluating TB and MDR-TB control activities in the MDR-/XDR-TB era, within the framework of the implementation of the new Stop TB Strategy, at national and sub-national level.
The target staff at the national or sub-national level is usually a physician (in Europe) or a nurse (in Africa and Asia) working at the Ministry of Health at a national or sub-national level, within the national TB control programme, national HIV/AIDS programme, or in National TB and HIV/AIDS Institutes. Usually he/she does not have clinical duties (or they have a part-time clinical job), the job being primarily administrative and managerial. Although the staff must be thoroughly familiar with clinical guidelines for the national TB control programme (NTP, as well as for the national HIV/AIDS programme, ACP or other related programmes, e.g. hospital infection control programmes), he/she is primarily responsible for enabling and
monitoring the implementation of these guidelines rather than applying them at the facility level. Therefore, the course was not designed to train staff managing or working in individual hospitals or health facilities. This course is essentially addressing the national/sub-national TB/MDR-TB control perspective more than that of the single hospital/health facility one. Furthermore, the course is not focused on the specific clinical aspects related to diagnosis and treatment of clinical cases. The Units dealing with clinical aspects are mainly focused at dealing with the public health aspects related to management of diagnosis and treatment activities at programme level.

The following tasks to be performed by the target population on the job were defined:
1. Plan and co-ordinate a mission with WHO/Contracting Agency, local authorities and other partners
2. Review data on the key components of NTP (using standard data collection tools, including the Planning and Budgeting tool) based on the new Stop TB Strategy
3. Analyse and synthesize the main findings, identify priorities, propose solutions and develop recommendations
4. Write a report including key information and recommendations relevant for present or future activities according to the Contracting Agency needs and the kind of mission
5. Debrief local staff and partners on the main mission’s findings and recommendations.
6. Submit the report to the Contracting Agency for finalization and planning of further actions/steps
7. Design a plan on MDR-/XDR-TB control and implementation of TB/HIV activities
8. Manage different steps of a GFATM proposal
9. Calculate ACH (Air Changes per Hour) in different rooms and identify priorities for infection control (with focus on high MDR-TB and HIV prevalence settings)

The skills and knowledge requested to perform the tasks were then defined.

D5.2: Course skeleton and training materials plan

Based on the task analysis, defining skills and capacity requested to perform the task, the learning objectives were developed by April 2009

The selected skills and knowledge were organized into suitable teaching units (modules) and the training design (including brief outlines of module content and planned training methods) was developed. The draft course curriculum was designed consisting of three parts and 28 units (Part 1: 4 units; Part 2: 14 units; Part 3: 10 units). A draft Participants’ manual was developed by the end of April 2009, in order to be tested during the May 2009 version of the training.

The course uses a variety of methods of instruction, including presentations, exercises, discussions, exchange of experience among facilitators and participants and a field visit to a local health facility offering TB and HIV/AIDS services. Practice, whether in problem-solving exercises, discussions, exchange of experience among facilitators and participants or in the health facility, is considered a critical element of instruction.

- Presentations are designed to introduce the topics of the units, discussions and/or exercises.

- Exercises are based on data from Fictitia, an imaginary country in which the expansion phase of DOTS is being completed that is facing the effects of a growing HIV/AIDS epidemic. Annex 1 of the manual for participants is a concise background document including the description of the country, its infrastructure, epidemiological and financial data as well as other information needed for the exercises.

- Other exercises include case studies, role plays, small group discussions, facilitated discussions and case analyses.
Other interactive activities are conducted to facilitate the full comprehension of new information and the development of skills. Presentations and discussions are based on selected country experiences (such as Malawi) and on the participants’ own experiences.

A field visit is organized to a health facility where services for people with TB/HIV are provided. Senior counsellors and health care staff from the health facility facilitate the visit.

Each unit of the participants’ manual provides the necessary information for participants on objectives, methods and materials. Slides and exercises are also reported at the end of each unit.

In order to allow reaction evaluation, as well as learning and performance evaluation an evaluation questionnaire was developed.

By April 2009 the evaluation questionnaire was developed and included in the Participants’ manual to allow reaction evaluation by May and October 2009 training participants. The questionnaire allowed also through its open question space to evaluate learning and performance evaluation. In addition learning and performance evaluation was performed by analysing the reports, the plans and the problems-solving exercises representing the outcomes of the training courses.

The course director, in consultation with facilitators scored the overall results of the training as excellent in a scale including insufficient, fair, good and excellent. This evaluation was done considering the following criteria: capacity to interpret the setting (“Fictitia”), capacity to identify and prioritise the problems, formulate and prioritise the feasible solutions the problems identified. In addition the facilitator panel took the quality of the group and class discussion following the panel presentation of the exercises into account.

D5.3: Standard PG courses and other activities of the European School of Respiratory Medicine

The ERS School’s Post Graduate Course (PG) was organized in order to train European respiratory clinicians and specialists on state-of-the-art management and control of MDR- and XDR-TB on the basis of materials and modules developed.

The PG 12 “Update in tuberculosis: advances in the management of drug resistant and multi-drug resistant tuberculosis cases ” was performed as planned during the ERS Annual Congress, in Vienna in September 2009. The aim was to update clinicians on the International Standards of TB Care; MDR-TB, and the WHO recommended strategy of TB Control; and updates on new diagnostic methods and treatment approaches. The target audience were respiratory physicians from Eastern Europe and CIS, and the ERS encouraged their participation by sponsoring attendance of delegates from these countries. It was a well-attended course with 75 participants and the course evaluation was very positive.

D5.4: Preliminary course materials on MDR-TB

The draft training course was pre-tested in May and October 2009. In order to allow a double testing of the training materials, a significant effort was done to complete the Participants’ manual ahead of schedule. After completing the evaluation of the two above mentioned training courses the drafting of the Facilitator and Course Director manual started in December 2009 and was completed in January 2010.

D5.5: Results from field tested training on MDR-TB

The training course “Implementing the Stop TB Strategy: skills for managers and consultants (TB, MDR and more)“ was field tested in May and October 2009. The evaluation of the courses was very positive:
about 80% scored the courses as excellent and 20% good.

D5.6: Final course materials

The version capturing participants’ comments was completed by January 31st, 2010. The evaluation procedure was submitted together with the entire training package to P13 for comments in February 2010. The training course material was completely revised capturing both participants comments and specialised comments by P13 received in March 2010 after review of the training package. The final course material was available and used for the Sondalo training course in May 2010.

D5.7: Training of physicians’ facilitators

The basic standardized innovative training material to be used to train the physicians’ facilitators has been developed within the framework of the Sondalo training course taking into account the participants’ and partner’s P13 comments.

D5.8: Standard training of TB managers

The training is being evaluated as planned during the Sondalo training course.
The training course for TB managers: “Implementing the Stop TB Strategy: skills for managers and consultants (TB, MDR and more)” has been performed and evaluated as planned in Sondalo in October 2010 (Mo22), in May 2011 (Mo 29), in November 2011 (Mo 35), in May 2012 (Mo 41), in October 2012 (Mo 46), in May 2013 (Mo 53), in October 2013 (Mo 58) and in May 2014 (Mo 65).

An extensive revision and update of the course material has been conducted to cope with the changes of the WHO Strategy occurred during the last year of the project. The new post 2015 WHO Strategy (approved in December 2013 by WHO and in May 2014 by the World Health Assembly after several rounds of discussion with countries, technical agencies and stakeholders- including FSM) promoted the concept of TB elimination, and the new vision of the strategy is now based on 3 new pillars (high quality, integrated TB care and prevention; bold policies and supporting systems; intensified research and innovation), the entire structure of the course performed in May 2014 has been revised to fit the new pillars. As a consequence, the following modules have been comprehensively revised: MDR (Unit 8), planning (16), clinical management (5), and community engagement (Unit 12) whose content was modified to cover new topics like addressing poverty, universal health coverage and social protection. After this extensive revision the course title was changed from “Implementing the Stop TB Strategy: skills for managers and consultants (TB, MDR and more)” to “Towards the WHO post-2015 global TB control and elimination strategy: skills for managers and consultants” be more consistent with the up-to-date course.

D5.9: Inclusion of innovative training modules into standard PG courses of ERS School

The innovative training modules and educational materials developed under D 5.7 have been captured in the PG12 “TB PAN-NET Postgraduate Course: ‘Train the trainer’ (Invite only)” performed during the ERS Annual Congress, in Barcelona (September 2010) generating standardised training materials to be used in future ERS PG and professional trainings.
further ERS school PG and educational events. The aim of the PG was to prepare qualified tuberculosis specialists from Eastern Europe and former Soviet countries, who afterwards will be able to carry out quality medical practice in their countries and to train others in how DR-patient management should be done.

Twenty-five delegates were personally invited to the session, selected among those who could be adequate as facilitators (i.e. well-known experts in the field of DR-TB, who would be ready to train other people using the materials they were shown). The course evaluation was very positive.

In addition the innovative training modules and educational materials have been captured in the first and second version of the Pulmonary TB chapter of the ERS Handbook part of the ERS-HERMES project (European Respiratory medicine certificate).

D5.10: Training of clinicians (PG course)

The following PG courses were performed during the ERS Annual Congress:
- PG12 “Multidrug-resistant and extensively drug-resistant tuberculosis: update on clinical management”, in Amsterdam, September 2011 (Mo33)
- PG7 “TB and MDR-/XDR-TB: what is new in diagnosis, treatment and follow-up (TB PAN-NET)” in Vienna, September 2012 (Mo45)
- PG7 “Tuberculosis: innovations in clinical and public health management (TB PAN-NET)” in Barcelona, September 2013 (Mo 57).

The facilitators were selected from the ones who received training during the ToT PG course in year 2 (D 5.9) and trained clinicians using the innovative training module and educational materials developed in D 5.7.

D5.11: Training of clinicians (external courses)

The primary objective of the ERS external courses was to train facilitators on MDR/XDR-TB using the evaluated modules and materials developed. Faculty was chosen from the ToT PG course; the educational material was provided in English and translated into Russian. The courses were exercise-based with ERS sponsoring participation to ensure representation from core High TB incidence European countries.

A total of three ERS external courses were organised.
- The first ERS external course “Tuberculosis at times of emerging drug resistance”, was held in Borstel, Germany in May 2010 (Mo 17) one year earlier than planned (Mo 33). As discussed with the coordinator, the course was held in a location (in terms of geographical location and flight connections) considered strategic to allow the maximum participation from Russian-speaking countries. In fact, twenty Russian-speaking people attended the courses and both English and Russian versions of the course book were made available. The number of participants was 80. The course evaluation was very positive.
- The second ERS external course “TB and M/XDR-TB: from clinical management to control and elimination” was held in Bucharest, Romania, in May 2012 (Mo 41) as planned. The course had 81 participants from 23 different countries. Participants from Romania and Latvia made up the largest group of attendees. The educational materials were translated into Russian. The course evaluation was very positive.
- The third ERS external course “TB elimination: dream or reality” was held in Dubrovnik, Croatia, 29 May – 1 June 2013 (Mo 53) as planned and had 39 participants from 23 different countries.
June 2013 (Mo 53). It was a well-attended course with 39 participants coming from 23 different countries, in particular from FSU countries. Speaker presentations and practical workshops received positive feedback.

D5.12: School seminars

Two School seminars were organized in the frame of meetings organised by local or national societies with ERS sending speakers who made more theoretical presentation as in the External courses, excluding the interactive section. This format represented an additional way to reach out to a wider number of participants based on locally organised events. The first ERS school seminar planned in Mo 36 was not performed and the funding allocated for this seminar was joined with that originally planned for the second seminar allowing to run a full high impact school seminar. The seminar “What is new in TB diagnosis and treatment in Europe”, was held during the Union Europe Conference in London, July 4, 2012 (Mo 43) as planned. The Union Conference had been specifically organized to attract delegates from Eastern European Countries to discuss European TB control priorities. 67 persons attended, from both eastern and western Europe. There were representatives from the majority of EU countries (including the Baltics and a robust delegation from the UK). Russian speaking delegates were from Russia, Belarus. Moldova, Romania, Ukraine, and some Central Asian Countries were also represented. Representatives of WHO, ECDC and KNCV also attended the session. The sessions were quality, and an interesting discussion followed the presentations. The second seminar “Improved diagnosis and new drugs: Is this enough to eliminate tuberculosis” was held Chisinau, Moldova in June 2013 (Mo 54). It was a successful well attended seminar. 92 TB doctors from Chisinau and representatives from all regions (about 1 out of 3) attended the seminar. Materials and presentations were in English with simultaneous translation in Romanian. Preparation and integration of local vs international presenters was made easier by the fact that 10 core Moldovian colleagues attended the Sondalo training courses previously.

D5.13: Report on training results and overall impact of training for five years at the TB PAN-NET final meeting

Final report summarising WP5 activities, main results and deliverables achieved was prepared and presented at the TB PAN-NET final meeting.

Milestone N. Milestone name Due date Comments
M17 Availability of final material for training on TB drug resistance and clinical management M18 Training package available
M18 Availability of final material for ERS external courses In Eastern Europe Countries M18 Training for external courses locally available

Work Package 6- TBNET: Clinical research on TB drug-resistance in Europe

WP6 LEADER: FZB

Partners Involved: P3 FZB; P6 ERS;
Results Achieved

D 6.1: Defining clinical relevant variables for MDR-TB infection to be included in databank.

During month 1-12 the variables of the database have been identified and defined, with the support of a panel of international experts from WHO, StopTB Partnership, KCNV, TBNET. This was support by an intense literature review. Variables cover the topics risk factors, resistance patterns, treatment regimens, adverse event, concomitant treatments, and clinical outcomes.

D 6.2: Establishing a databank.

A tender was released for the design and management of the database. The Center for Poverty-related Communicable Diseases at Amsterdam Medical College (now Amsterdam Institute for Global Health and Development) won the tender in July 2009. Different options regarding the data collection tool were considered. According to the requirements of the project “Open Clinica”, an open source clinical data management software was selected for the data collection and management. The database underwent several phases of design and pretesting. It was finally installed at the web server of the Research Center Borstel in January 2010. It contains a training module and a data collection module. Additional to a study protocol a code book and a data entry manual have been designed. The online database was constructed and pilot tested between months 7–13. Several technical challenges regarding the hardware and the software installation had to be overcome (definition of variables and database construction). Therefore potential partner institutions for the data collection have been contacted, but the live demonstration of the software and the distribution of the sampling platform could therefore only commence at month 13. The cost analysis was deemed not feasible due to huge variability in programme structure in countries, lack of availability of information in the target group of data collectors. A different approach is needed to obtain the consisting information. In 2012 we started the data collection of the cost of drugs for tuberculosis treatment from all European countries in order to evaluate the cost of drugs contributing to the total cost of treatment of drug resistant tuberculosis.

D 6.3: Distribution of study objectives

D 6.4: Distribution of sampling platform.

A detailed protocol of the study was prepared. During months 3 - 12 potential partners in the project (members of TBNET and new partners) were screened and contact to discuss a potential participation. Starting at month 13, the database has been presented to potential partners in all EU – countries plus TBNET partner institutions in Moldova, Turkey, Ukraine and Belarus, who have been invited to participate in the data collection. Due to logistical reasons, human resources and different levels of interest in the project, only a selection of institutions in several countries demonstrated interest in project. The role out of the project required considerable time, mostly for logistic reasons of obtaining ethical approval and establishing/finding a suitable partner and infrastructure to implement the project. Ethical approval has been obtained according to local requirements in the participating institutions, but was also a reason for delay or cancelling the implementation of the study, as the process was too long or too difficult (i.e. Finland).

Due to logistical reasons and limited internet access a paper- based version of the case record forms has been designed and is used for data collection in Latvia, Estonia, Romania and Moldova.
Various sites approached declined participation due to the workload of the project for clinicians expected and the anticipated impossibility to follow up patients after discharge from the treatment in the hospital during the intense phase of treatment. This limited the number of sites and increasing the sample size of the project.

In order to discuss the progress of the study, a core advisory group, consisting of the work package leader, the project coordinator and TBNET members has been formed. Regular conference calls have been held to discuss progress.

D 6.5: Training study coordinators

At all sites prior to implementation of the study a visit by the study coordinator was scheduled to present project details and to implement the study, considering site specific set ups and work flows. The complexity of the study objectives, definitions of variables and use of the database required a face to face training and intense monitoring of data collection and entry throughout the time of the project.

In May 2010 key partners from the sites in Moldova, Romania, Estonia and Spain participated in the annual TB PAN-NET meeting in Lübeck and had individual progress meetings with the work package leader and the project coordinator. A progress meeting between the chief epidemiologist, the data base developer, the data manager and the project coordinator was held in June 2010 in Amsterdam.

In order to support, correct and monitor the early phase of data collection, the project manager revisited the key sites in Moldova after month 9, 15 and 32 months of data collection, in Romania after month 3, 12 and 24 months of data collection and in Spain also after month 3 and 18 month. Follow up visits in Latvia and Estonia took place in January 2012. A visit to facilitate data entry at the site in London took place in November 2011. The study nurse performed visits in Parsberg, Groningen and Minsk in 2012, while the coordinator travelled to Riga, Tartu, Tallinn, Chisinau, Balti, Barcelona and Minsk for follow up visits.

During 2013 follow up visits in Chisinau, Balti, Bucharest, Vienna, Groningen, Nejmegen, Sondalo and Rome were performed, as the local investigators were not able to complete task on there own and data quality had to be improved by monitoring source data and improvement of completion.

A key event within WP6 has been the midterm meeting and workshop in Borstel on September 05th and 6th 2011. 32 investigators from all sites and speakers participated to discuss progress and challenges of the project. The meeting was also used for case discussion, presentations on challenging topics of M/XDR- disease care and for team building. Distinguished speakers from WHO (Dr. Masoud Dara) and ECDC (Dr. Andreas Sandgren) contributed with their presentations to the meeting.

Based on the success of the meeting in 2011 a follow up meeting was organized in 2012. On 24/25th of September 2012 participants from almost all sites participated in the progress review and discussions, as well as in the scientific symposium. We were able to attract speakers like Dennis Falzon (WHO HQ), Francis Varaine (MSF) and Martin Grobusch (University of Amsterdam).

Follow the overall success of the previous meetings in improving the outcome of the data base, motivating participants and discussing research progress, a final meeting was organized in November 2013. Participants from 12 countries discussed the results in team discussions and help interpreting the outcome of the study. Presentation from Frank Cobelens (KNCV), Carole Mitnick (Harvard University) and Michael Hoelscher (LMU Munich) stimulated the scientific discussion on diagnosis and management of M/XDR-TB and the visits consolidated partnerships i.e. with RESIST- TB and Amsterdam Institute of Global Health Research. A patient representative from the the Republic of Moldova gave the audience deep insides in the needs and suffering of our patients.
The meetings improved considerably the motivation, the team aspect and the knowledge of the investigators. Personal contacts are essential in a large collaborative project like the data base in order to motivate the participants. Based on the information and results of the meetings the intense data monitoring and review process could be revised and optimized, giving new guidance to the investigators to close gaps in the data completeness.

D 6.6: Data collection.

Data collection has been started at month 14 after pilot testing of the database, and was rolled out in 13 countries. The complexity of the database and the use of various case record forms as well the logistical challenges at each site, particularly due to the inpatient and ambulatory phase of treatment required a training and implementation visit at each site. Logistical issues, use of data, reimbursement and workflow had to be discussed and agreed on with the relevant stakeholders and partners on site. A training module in form of an overview presentation and a detailed training of data collection and entry have been designed and used during the mainly classroom training sessions. A codebook and a data entry manual, which has been designed, supports the training and data collection.

Due to the complexity of the database and its structure the focus of the project is on the prospective data collection currently, while retrospective data collection has only been implemented in one site currently. In June 2010 a data manager has been recruited, whose task is the quality control of introduced data and the feedback of missing data information to the participants. Two meetings between the project coordinator and the data manager have been held in 2010. In June a detailed feedback mechanism for the participants was designed and introduced.

Problematic is the fact, that patients with drug-resistant tuberculosis are initially treated in a hospital based setting and transferred later to a ambulatory setting. The transfer of the patients also requires a transfer of the data collection procedure to the outpatients setting. This poses many logistical challenges for the data collection and requires additional human resources to fulfil the task. In order to support the partners at the sites in completing data entry and maximizing data quality a study nurse has been employed on part time basis to support this activities.

At total of 380 M/XDR- TB cases and 376 non M/XDR- TB cases were completed in a format to be included in the final cohort analysis. The employment of the study nurse strongly enhanced the quality and completeness of data, as support at the study sites to complete tasks was deemed necessary.

An intense review and monitoring process, including automated checks and manual plausibility checks during month 52 to 62 strongly supported the high quality completion of the data collection.

D 6.7: Data analysis

Data analysis is done in cooperation with the epidemiologist in the project, who is based at Amsterdam Institute of Global Health in the Netherlands. Regular phone conferences are used, to prepare, perform and discuss the details of the data analysis.

For the purpose of the midterm meeting in September 2012 an analysis of baseline data has been performed. Baseline indicators like gender, residence, country of birth, cohort, age distribution, resistance pattern have been analysed after a midterm deadline for data entry on July, 31st 2011.

A meeting at took place on May 2nd /3rd of the data analysis team in order to plan the details of analysis and a publication strategy. In April 2013 a 2 day retreat of the core team defined details of the data
analysis plan and strategy.
Interim analysis was done on the occasion of a invited presentation of WP results at the ERS Scientific meeting in Barcelona in September 2013 and the investigator meeting of TBPANNET in November 2013 in Borstel.
Following the final investigator meeting in November 2013 the publication strategy was revised and co-authors from the group of investigators identified.

The following papers are envisaged.
1st manuscript - demographics of MDR patients and Europe including comorbidities/risk factors/migrants - comparing MDR/non MDR
2nd manuscript - Risk factors for FQ, Injectable, XDR
3rd manuscript – 3months/6months conversion related to DST and treatment
4th manuscript – adverse events in the first 6 months of treatment
5th manuscript cost of drugs and availability of drugs
6th manuscript Variability of management, adjuvant treatments and surgery
7th manuscript Use of dynamic regimen in drug-resistant tuberculosis
8th manuscript Management of HIV/TB confection in Europe
9th manuscript Risk factors of treatment failure in a cohort of TB in Europe
10th manuscript Treatment outcomes of susceptible tuberculosis in Europe
11th manuscript Dosing of TB drugs in a European cohort

Manuscript 1 was completed following the conclusion of data collection in March 2014 and submitted in May 2014. Manuscript 5 was also submitted in June 2014. Manuscript 2 is in preparation.
Additionally at all sites, where possible, DNA from patients included in the MDR-TB cohort were obtained in order to

D 6.8: Final data evaluation.

The final data evaluation is a ongoing process beyond month 66 of the project. The reason is, that the data collection and monitoring process was only completed due to the length of the treatment process of patient with M/XDR- TB in month 63. The data evaluation goes along with the publication of the results according the strategy described in D. 6.7.
The cooperation in the WP6 with the site in Bucharest led to a sustained partnership, building now a study center with the German center for infection research. The partnership with the National TB programm in Moldova led to further activities, like joint PhD programmes on TB related topics, teaching activities at the Medical Faculty in Chisinau and further planned joint research projects.

Milestone N. Milestone name Due date Comments
M19 Establishment of data collection infrastructure M8 Software released
M20 Evaluation of interim analysis and amending the research focus if necessary M30 Report from the meeting

Work Package 7- Dissemination and information sharing

WP7 LEADER ERS
WP7 LEADER: ERS

Partners Involved: P1 USR; P6 ERS; P29 FCSR;

Results Achieved

D7.1: TB PAN-NET Specific project website made available via the ELF website

The project website was created in 2009. The site provided constantly updated information to the TB community including information such as:

1. Announcement and highlights of different events such as meetings and courses.
2. Upload of educational material from courses organised in the framework of the FP7 including postgraduate courses, in addition to other interactive modules and educational material from ERS database.
3. Latest news related to MDR/XDR-TB based on the European Lung Foundation Factsheet. The content was translated into Hungarian, Polish, Romanian and Russian. The ERS provided support to the project partners by updated information on request. The ERS plans to continue hosting the site in the near future.

D7.2: Set-up web-based communication platform for restricted access for community including member-emailing lists for access to internal meeting reports; technical dossiers; internal project reports

The web based communication platform was set up in month 2. The platform ways accessible from the members section of the tbpannet.org website. The software initially used was adobe connect. In the 2012 this technology was replaced by ww.box.com to allow greater collaboration.

D7.3: Kick-off press release


D7.4: ELF fact sheet written in lay terms on MDR/XDR-TB. To be made available via the ELF website, translated into different Eastern European languages (e.g. Polish, Hungarian, Romanian, Russian etc)

Available on TB PAN NET project and ELF websites. Translation into 10 languages fully completed.

D7.5: Translate parts of the project website introducing the project rationale, objectives etc into Eastern European language

The entire website was translated throughout the project into Russian, Polish, Hungarian, English and Romanian.

D7.6: ERS Congress: Session at TB symposium

The scientific symposium “Tuberculosis drug resistance and migration” (350 participants) was organised by ERS and TB PAN NET project. Reviewing drug resistance and migration.
at the ERS Annual Congress in Barcelona and was chaired by Dr. C. Lange and Dr. G. Sotgiu.

D7.7: ERS Newsletter: dissemination on project developments

Newsletters were sent out regularly (every 3-6 months) to provide updates to the TB PAN NET community.

D7.8: ERS Congress: Session on TB Symposium

The scientific Symposium on “MDR-/XDR-TB: update on clinical management” was organised at the ERS Annual Congress in Amsterdam, 26 September 2011 (>400 participants; 30 countries represented; 4.43 out of 5 average speaker mean). The symposium provided an updated on guidance for the implementation of TB care in the EU on WHO guidelines for the management of drug resistant TB.

D7.9: ERS Congress: Session/symposia TB

In collaboration with P4-FSM, Tradate (IT), organisation of a symposium at 2013 ERS Congress entitled the Best of TB PAN NET.

D7.10: ERS Stakeholder Workshop on MDR-TB

In collaboration with USR (P1) the organisation of the final stakeholder meeting in Brussels, Belgium, on 1 April, 2014.

Milestone N. Milestone name Due date Comments
M21 Annual scientific meetings M1, 12, 24, 36, 48, 60 Abstract available
M23 Final dissemination workshop M66 Abstract CD/book

Potential Impact:
The importance on Pan-European Research network is very well known at the European level and TB PAN-NET clearly represents an example in terms of goals achieved and objectives met. The project has produced considerable impact on the related industry and research disciplines, and it has achieved largely all the planned deliverables in target timeline. During its lifetime the project was extremely successful on many aspects. We showed the value of large collaborative projects including Academia, Research centres, public Health Institutions, SME, Scientific Societies and Hospitals with different professional profiles and expertise. We also showed the potential of sharing data in large combined databases in the TB field. Data on typing results with the new generation sequencing, especially including hypervariable loci mycobacterial interspersed repetitive units (MIRU), open up to new studies to better understand cross-border issues in the continent. The innovative geo-platforms potentially provide immense clues in the understanding of the epidemiology of TB in the countries and further targeted control of the disease. Likewise, the findings from the study of Beijing lineages on a global scale are very important and insightful, especially the cross-border clonal expansion complexes. Such illustration of the bacillary genetic diversity helps in the enhanced understanding of evolution of the genotypes. Furthermore, complementing the

<table>
<thead>
<tr>
<th>Milestone N.</th>
<th>Milestone name</th>
<th>Due date</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>M21</td>
<td>Annual scientific meetings</td>
<td>M1, 12, 24, 36, 48, 60</td>
<td>Abstract available</td>
</tr>
<tr>
<td>M23</td>
<td>Final dissemination workshop</td>
<td>M66</td>
<td>Abstract CD/book</td>
</tr>
</tbody>
</table>
molecular findings with clinical data would be of enormous value in helping the interpretation of the molecular data in transmission dynamics, and subsequently in planning intervention strategies to curtail the spread of drug-resistant disease, within European countries and geographical areas and beyond. SNPs profile generated by NGS represents the future of molecular diagnostic also for TB. However, several gaps remain to be filled, including 1) the capability to discriminate high confidence SNPs responsible for DR from compensatory or phylogenetic SNPs; 2) the capacity to predict cross resistance to different generation of molecules belonging to the same antibiotic class, providing information for personalized therapy 3) development of clear and clinician friendly interpretation rules correlating SNPs to their clinical prognostic value 4) developing specific protocols to analyse only nucleic acid derived from live bacteria. In this view, WP2 can be considered a precursor of actual needs: in this WP we provided large databases for SNPs and their clinical relevance in patients’ management and phenotypic resistance. In addition, we provided novel drug resistance pathways and players (e.g. smallRNAs), thus contributing to forthcoming NGS-based work. Developing mono-R strains, we provided accurate gold-standard strains to be used for EQA. We developed novel approaches to apply molecular testing for treatment monitoring, excluding the signal derived from dead bacteria. Finally, we provided novel insights also on the host’s side studying human miRNAs as novel players during the infection and as novel biomarkers.

An important line of work has been devoted to the promulgation of the concept and practice of external quality assessment (EQA) system for phenotypic drug susceptibility testing (DST) inclusive of second-line anti-tuberculosis drugs. A large multi-center study, defining the accuracy of different phenol- and genotypic drug susceptibility methods for 1st and 2nd line drugs are now available. The final results of WP3 will help to get the answer, which methods provide the clinicians with the most accurate, reliable results, necessary for an individualized treatment regimen. The development of mono-resistant strains is a major step in performing external quality control under safe conditions. Additionally, WP3 identified the best method to detect heteroresistance, also very important for the establishment of a successful personalised treatment. Rifampicin is the most important drug for therapy of tuberculosis and resistance should be detected properly and easily by different methods, including molecular based techniques. We evaluated the performances of the different methods currently in use. In summary the results of WP3 provide the clinicians with fast and reliable results for 1st and 2nd line drugs, which is very important not only for the proper treatment of the individual patients but also for the impact on stopping the transmission of highly resistant strains in the community.

A number of very robust clinical and diagnostic studies resulting in a profusion of scientific publications underpin the achievement of the Work package 4. A multi-centre study provided valuable information on risk factors for MDR/XDRTB primary infection as well as resistance acquisition during treatment that can guide public health policy as well as serve as an exercise for establishing an integrated and synergistic network for future research. Large biobank of host and pathogen biomaterial with the relevant clinical and epidemiological information available presents a valuable research resource for future research including discovery of yet unknown biomarkers in future studies and allows to perform detailed assessment of novel candidate biomarkers. Furthermore, biomarker studies, currently in progress would potentially allow to identify patients not responding to treatment at an early stage and enable clinicians to promptly adjust treatment regimen accordingly.

The analysis of the dataset on clinical variables about drug-resistant tuberculosis contributed to the creation of a wider evidence base for the management of drug-resistant tuberculosis. Availability of an adequate number of specifically trained human resources is considered by all NTPs key
point for a successful management of MDR-TB cases. During the first two years (2009-2010) of the FP7 TB PAN NET project a standardized innovative training material has been developed and annually updated to train in Sondalo. At the end of the project, 281 managers and opinion leaders involved in MDR-TB programmes in Europe and in high burden countries responsible of TB migration into Europe have been trained. The basic standardized materials developed within the framework of the Sondalo training course are going to be used to train the physicians' facilitators. The educational materials developed have been captured in the first and second version of the Pulmonary TB chapter of the ERS Handbook (ERS-HERMES project) contributing to the education and training of the European clinicians and specialists (used by European physicians to pass the Hermes European examination in Respiratory Medicine) and in the new ERS advocacy initiative, the White Book launched in September 2013.

ERS PG courses and educational events organised on the basis of the training materials and modules developed will contribute to prepare and educate young respiratory clinicians dealing with TB. WP 6 has investigated the clinical course of MDR-TB and non-MDR-TB at 23 sites in 15 countries. The results describe important details for the clinical and public health analysis of MDR-TB and inform other stakeholders and health policy makers about the challenges of TB management at the frontline of patient contact and decision making towards cure of individuals and control of transmission. But WP also allowed training clinicians and establishing operational research at sites, where clinical research was rarely performed, as Balti and Chisinau in Republic of Moldova, Bucharest in Romania and Minsk in Belarus. Based on the collaboration and training, future projects are envisaged. Further the workshops performed with study participants from Eastern and Western Europe and the participation of international experts allowed education and discussion of diagnostic and treatment strategies between clinicians from European MDR-TB treatment centers in a new format. This has led to new cooperation and exchange processes in the management of particularly of migrating TB-patients in Europe. The results of the study will describe in detail a clinical cohort of non-MDR-TB patients, as rarely done in the last decade. Further the results of the MDR-TB cohort are the obtained almost entirely before the introduction of new anti-TB drugs and will therefore be a good comparison to new treatment strategies in the future.

List of Websites:

http://www.tbpannet.org/
Beneficiary
No.
Beneficiary
Name Beneficiary
Short name Country E-mail
1.
Coord. Università Vita-Salute San Raffaele USR Italy cirillo.daniela@hsr.it

2. Queen Mary and Westfield College, University of London HPA-MRU United Kingdom
   f.drobniewski@qmul.ac.uk
3. Forschungszentrum Borstel, Leibniz-Zentrum für Medizin und Biowissenschaften FZB Germany
sruesch@tz-borstel.de
4. Fondazione Salvatore Maugeri FSM Italy gbmigliori@fsm.it
5. Azienda Ospedaliera San Gerardo di Monza AO S. GERARDO Italy andrea.gori@unimib.it
6. European Respiratory Society ERS Switzerland fernando.martin@ersnet.org
7. Foundation for Innovative New Diagnostics FIND Switzerland mark.perkins@finddiagnostics.org
8. Swedish Institute for Communicable Disease Control SMI Sweden sven.hofner@smi.se
9. Statens Serum Institut SSI Denmark esn@ssi.dk
10. Croatian National Institute of Public Health CNIPH Croatia v.katalinic-jankovic@hzjz.hr
11. Università degli Studi di Siena UNISI Italy pozzi@unisi.it
12. Hain Lifescience GmbH Hain GmbH Germany david.hain@hain-lifescience.de
13. (terminated) State Agency Infectology Centre of Latvia Clinic of Tuberculosis and Lung Diseases LIC Latvia Ludmila.Viksna@rsu.lv
14. “Guido Montessori” Company Montessori Italy montessori@cogeor.com
15. Tartu University Hospital TUH Estonia Manfred.Danilovits@klinikum.ee
-16. (UTRO) National Tuberculosis and Infectious Diseases University Hospital NRL Lithuania Lithuania edita.david@takas.lt
16. Vilnius University Hospital Santariskiu Klinikos VULSK Lithuania edita.david@takas.lt
17. University of Vilnius, Institute of Biotechnology VU IBT Lithuania pstak@ibt.lt
18. Norwegian Institute of Public Health NIPH Norway turid.mannsaker@fhi.no
19. University Clinic of Respiratory and Allergic Diseases Golnik NRL Slovenia Slovenia manca.zolnir@klinikagolnik.si
20. Institute of Tropical Medicine ITM Belgium irigouts@itg.be
21. Spitalul de Pneumoftiziologie Brasov SPF Brasov Romania iosomun@gmail.com
22. Marius Nasta Institute of Pneumology I-M.NASTA Romania cristoprea@yahoo.com
23. Institut Pasteur de Lille IPL France philip.supply@ibl.fr
24. National Institute for Health and Welfare THL Finland merja.marjamaki@thl.fi
25. Scientific Institute of Public Health - Institut Pasteur IPH Belgium vmathys@wiv-isp.be
26. National Tuberculosis and Lung Disease Research Institute NRL Poland Poland e.kopec@igichp.edu.pl
27. University of Medicine and Dentistry of New Jersey UMDNJ New Jersey, USA gennarma@njms.rutgers.edu
28. Riga Eastern Clinical University Hospital REUH Latvia Ludmila.Viksna@rsu.lv
29. Fondazione Centro San Raffaele FCSR Italy cirillo.daniela@hsr.it

Last update: 8 April 2015
Record number: 159240