PROJECT FINAL REPORT

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4.1 Final publishable summary report

Executive Summary

Infectious diseases by opportunistic pathogens such as *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* retain a prominent position as a major worldwide cause of morbidity and mortality in a wide range of patients. This problem has worsened with the emergence of antibiotic multi-resistant bacteria and the failure of drug discovery programmes to design antibiotics with truly novel modes of action. NABATIVI was supported within the programme FP7-HEALTH-2007-2.3.1-1 with the objective to **identify and validate novel drug targets** in order to **select lead compounds** for future development of a new class of anti-infective drugs against Gram-negative bacteria. Two approaches have been considered:

A) Discovery Phase Approach A: "From target to lead compound". A combination of advanced **genomic approaches** devised to screen the entire genome of *P. aeruginosa* for the identification of potential targets was applied from the beginning. Essential and virulence bacterial targets (genes) have been identified and validated in clinical strains and in a sequential cascade of disease models including *Caenorhabditis elegans, Drosophila melanogaster,* human cell lines and mouse models. These models have provided the rationale and the proof-of-concept for the characterisation and validation of selected **targets as candidates** for the development of antibacterials. Amongst the different targets, we focused our attention on i) quorum sensing systems, which orchestrate the expression of different virulence factors and have the potential to substitute or complement traditional antibiotic treatment; ii) essential cellular processes including the bacterial cell wall; iii) virulence factors which were not recognised in previous discovery programmes.

Using a combination of targets both previously known and discovered during the NABATIVI project, several screening processes (*in silico* and functional) were used and a number of **potential lead compounds** identified from both synthetic and natural compound libraries. These compounds will have to be further profiled to assess their therapeutic potential. In addition, a number of extracts from natural products and peptide nucleic acids (PNAs) have been found to inhibit some of these targets. The identification of the active compounds from these is under way but may run beyond the lifespan of NABATIVI.

B) Discovery Phase Approach B: "From drugs to targets". In this case it was the **natural antimicrobial peptide protegrin I** which served as starting point to discover a novel class of antibiotics with a novel mode of action. The target (gene) was identified during the drug discovery process. In particular we have identified **LptD** as **novel drug target**. LptD is an outer-membrane protein widely distributed in Gram-negative bacteria. The LptD–drug lead interaction causes a major disturbance in the outer membrane structure, possibly by interfering with its biogenesis, which requires LPS. Hit-to-lead and lead optimisation following a multidimensional optimisation strategy of ADMET properties led the novel compounds POL7001 and POL7080, which are effective against a wide range of clinical strains. Protection against lethal *P. aeruginosa* infection with potency superior to currently available antibiotics in preclinical studies led to **POL7080**, which was nominated as a **clinical candidate**. On 4th of March 2013 Polyphor Ltd announced the successful completion of a Phase I clinical trial demonstrating the clinical safety and tolerability of its Pseudomonas specific antibiotic POL7080. All primary study objectives were achieved in this Phase I trial. The study was a randomised, double-blind, placebo-controlled Phase I dose escalation trial assessing the safety, tolerability and pharmacokinetics of POL7080 in 52 healthy male volunteers. The study confirmed that this novel antibiotic was well tolerated by healthy volunteers and thus complements the comprehensive safety and tolerability

profile of POL7080. Polyphor expects that POL7080 in Phase II trials will demonstrate its efficacy in treating lung infections caused by multi-drug resistant Pseudomonas bacteria, in line with guidance from the health authorities. On 4th of November 2013, Polyphor Ltd and Roche announced that they have entered into an exclusive worldwide license agreement to develop and commercialize Polyphor's investigational macrocycle antibiotic POL7080 for patients suffering from bacterial infections caused by *P. aeruginosa*.

Overall, work by NABATIVI has opened a window for the design of novel antibiotics with novel mode of action and provided an excellent example of how academia and a small innovative company can work productively together to fill the gap left by the migration of big pharmaceuticals away from antibacterial field.

Project Context and Objectives

Antimicrobial resistance (AMR) is an increasing, global problem for public health. We are in the midst of an emerging crisis of antibiotic resistance for microbial pathogens in the EU and throughout the world (http://www.idsociety.org/badbugsnodrugs.html). Highly virulent and increasingly AMR pathogens have been described in different contexts, including the hospital and in the community. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death. The death rate for patients with serious infections treated in hospitals is about twice that in patients with infections caused by non-resistant bacteria. When infections become resistant to first-line medicines, more expensive therapies must be used. The longer duration of illness and treatment, often in hospitals, increases health-care costs and the economic burden to families and societies.

The global **human burden** posed by drug-resistant infections is difficult to quantify, but we have reason to fear that it may be enormous. Antibiotic-resistant germs are now found regularly in many hospitals in the EU, infecting some 4 million patients every year (focusing only on a limited group of healthcare-associated bacterial infections the burden is in the range of 2.5 million hospital days), causing 25.000 deaths and economic losses in the order of €1.5 billion due to extra healthcare costs and productivity losses (http://ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?ID=444).

Each year, in EU countries...

25,000 deaths directly attributable to multidrug-resistant infections

Extra in-hospital costs	Extra outpatient costs	Productivity losses due to absence from work	Productivity losses due to patients who died from their infection	TOTAL
€ 927.8 million	€ 10 million	€ 150.4 million	€ 445.9 million	**€ 1.5 billion**

Source – *The bacterial challenge: time to react*, Joint Technical Report from ECDC and EMA, Stockholm, September 2009. Available online at: http://ecdc.europa.eu/en/publications/Publications/Forms/ECDC_DispForm.aspx?ID=444

**Estimates for 5 common multidrug-resistant bacteria (EU Member States, Norway and Iceland, 2007)*

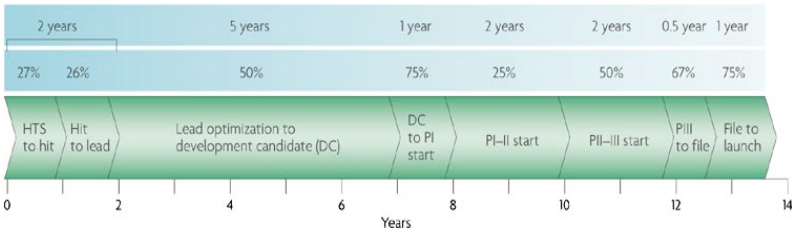
The most dangerous multi-drug resistant (MDR) bacteria were described as the **ESKAPE pathogens** including ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter* species. In particular, ***P. aeruginosa*** is a major and dreaded cause of infection causing a wide range of diseases in humans at various body sites including the respiratory tract, skin and soft tissues, the urinary tract, post-operative and burn wounds, brain, heart, bloodstream and cornea. Infections caused by *P. aeruginosa* are often life threatening and they are of particular concern for intensive care units (ICU) where ventilated patients may develop ventilator-associated pneumonia (VAP) and sepsis. Cystic Fibrosis (CF) is another disease where *P. aeruginosa* lung infections are frequent and often life threatening. Current treatment regimes of *Pseudomonas* infections either involve broad-spectrum antibiotics such as fluoroquinolones or aminoglycosides, carbapenems such as imipenem or meropenem, third generation cephalosporins such as ceftazidime, or drugs such as the monobactam aztreonam and the penicillin derivative piperacillin, either alone or in combination with tazobactam. However, frequently observed inefficacy of these treatments is linked to intrinsic resistance of *P. aeruginosa*, development of antibiotic resistance and/or limited penetration of antibiotics into biofilms (Gellatly, 2013).

AMR is a complex problem driven by many interconnected factors, so single isolated interventions have little impact and coordinated actions are required. According to the WHO

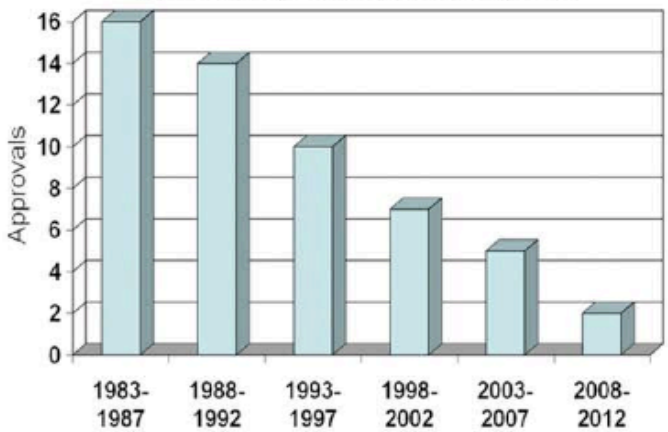
(http://www.who.int/en/), factors that accelerate the **emergence and spread** of AMR include:

- lack of a comprehensive and coordinated EU response;
- weak or absent antimicrobial resistance surveillance and monitoring systems;
- inappropriate use of antimicrobial medicines;
- poor infection prevention and control practices;
- insufficient diagnostic, prevention and therapeutic tools
- insufficient research and development activities due to the lack of federal and private funding;

In spite of the pressing need for new drugs to treat multidrug-resistant bacterial infections, there are simply not enough new antibiotics in the **pharmaceutical pipeline** to keep up with the pace of emerging resistance. Of greatest concern is the departure of many large pharmaceutical companies from antibacterial drug discovery and their decreasing investment in this area of research (Spellberg, 2004). The length of time (~10 to 15 years) and huge costs (U.S. $800 million on average) associated with the taking of a new drug from the discovery phase to market (DiMasi, 2003), combined with the perceived failure of whole-genome sequence-based approaches to spur a second golden age of novel antibacterial drug classes have led many companies to prioritise other areas of research (Fernandes, 2006). For many reasons, investment in the discovery of antibacterials is not as attractive to companies as research into other novel therapeutic drugs. First, the success rate for the discovery of drugs in other therapeutic areas is four to five times higher than that for antibacterial discovery, according to the GlaxoSmithKline screening metrics (14 high-throughput screening runs are required to obtain one lead compound) (Payne, 2007). Since multidrug resistant infections are still a small proportion of the total, although serious for suffering patients, the market for antibiotics is small, and is restricted in poor countries that cannot afford the costs. Finally, repeated prescriptions are unnecessary, and the treatments are short in comparison to therapies for chronic long-term pathologies, representing a disadvantage for drug



New systemic antibacterial agents approved by the US Food and Drug Administration per 5-year period, through 2012.



Antibacterial Pipeline (Anti-Gram Positive and Anti-Gram Negative),

Company	Since 1998	Phase 2/3
Abbott Laboratories	0	0
AstraZeneca	0	2
Bayer	0	0
GlaxoSmithKline	0	1
Lilly	0	0
Merck/Schering-Plough	1	1
Novartis	0	0
Ortho McNeil/Johnson & Johnson	1	0
Pfizer/Wyeth	2	0
Roche	0	0
Sanofi	0	0

Boucher H W et al. Clin Infect Dis. 2013;56:1685-1694

companies. Thus, the number of new antibiotics annually approved for marketing in the United States has continued to decline. Importantly, the number of large multinational pharmaceutical companies (i.e., “Big Pharma”) actively developing antimicrobial drugs also continues to decline. Only 2 new antibiotics have been approved since IDSA's 2009 pipeline status report (Boucher, 2013).

A **major limitation** in antibiotic development has been the difficulty associated with the identification of new structures that display the same low cytotoxicity for the host characteristic of conventional antibiotics, but at the same time have a narrow spectrum. No new classes of antibiotics were produced in the 37 years that elapsed between the introduction of nalidixic acid (a bacteriostatic quinolone marketed in 1962) and that of linezolid (Zyvox; Pfizer) (an agent used to treat infections caused by multidrugresistant Gram-positive bacteria marketed in 2000), which was followed by daptomycin (Cubicin; Cubist) in 2003 (Leeb, 2004) and, more recently, by retapamulin (Altabax/Altargo; GlaxoSmithKline) (Coates, 2002). All of the antibacterial agents that entered the market during this period were modifications of existing molecules (Boucher, 2009). Hence, physicians urgently need in their arsenals elusive antibiotics with novel structures and/or modes of action (Projan, 2002).

To overcome the inertia that currently surrounds antibiotic resistance, the **European Union** adopted a **strategic plan** on AMR based on a multifaceted approach. The EU's 6th and 7th Framework Programme fund a wide range of projects focusing on basic research, strategies for the prudent use of existing antimicrobials, development of new antimicrobials, development of point of care diagnostic tests, and vaccine development (http://ec.europa.eu/research/health/infectious-diseases/antimicrobial-drug-resistance/index_en.html).

One priority of the area of antimicrobial drug resistance within FP7-HEALTH is the **discovery of novel and efficacious antimicrobials** for treatment and prevention of bacterial infections. In particular, FP7-HEALTH-2007-2.3.1-1 has supported novel targets identification for drugs against MDR bacteria.

2.3. TRANSLATIONAL RESEARCH IN MAJOR INFECTIOUS DISEASES: TO CONFRONT MAJOR THREATS TO PUBLIC HEALTH

2.3.1. Anti-microbial drug resistance including fungal pathogens

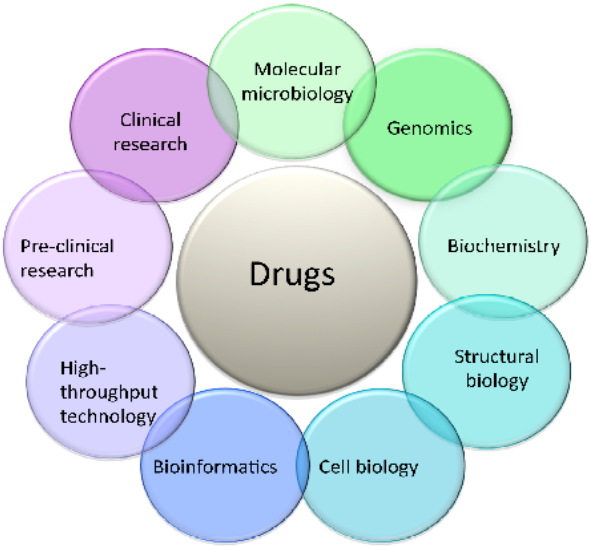
2.3.1-1: Novel targets for drugs against Gram negative bacteria.

The objective is to identify and validate novel drug targets in order to select lead compounds, which may be derived from natural sources or from synthetic compounds, for future development of a new class of anti-infective drugs against Gram-negative bacteria. Significant industrial involvement, particularly by SMEs, is foreseen in this topic.

Funding scheme: Collaborative projects (Small or medium-scale focused research projects with maximum EC contribution of € 6,000,000/project).

NABATIVI is a cooperative project supported within the programme FP7-HEALTH-2007-2.3.1-1. NABATIVI was specifically designed to provide key answers and mobilised the relevant know-how and resources needed to generate

new antimicrobial drugs by bypassing current antimicrobial mechanisms of resistance in Gram-negative bacteria. The research project was mainly focused on *P. aeruginosa* as a model pathogen, since it is an important, intrinsically resistant Gram negative bacterium responsible for high infection rates in humans within the hospital environment, has a completely sequenced genome and is highly amenable to genetic manipulation. However, the ubiquitousness of this organism had made the new discoveries potentially applicable to other important human pathogens such as *B. cenocepacia*, which was the secondary focus of this proposal.



NABATIVI brings together a multidisciplinary team including nine leading European research groups with complementary expertise to develop new drugs which could be exploited for the prevention and treatment of infections caused by *P. aeruginosa* and other gram-negative bacteria. The collaboration incorporates top-level expertise in the virulence of *P. aeruginosa* and related bacteria, in molecular microbiology and genetics, genomics, biochemistry, structural biology, drug development, analytical chemistry, structural biology, cell biology, bioinformatics, clinical research, epidemiology, and high-technologies related to the high-throughput screenings of compound libraries.

Six academic groups and three small innovative companies have worked productively together to fill the gap left by the migration of big pharmaceuticals away from antibacterial development.

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Main Scientific & Technological Results

The objective of NABATIVI was to identify and validate **novel drug targets** in order to select **lead compounds** for future development of a new class of anti-infective drugs against **Gram-negative bacteria**. This objective impacts on the increasing emergence and spread of antimicrobial drug resistant pathogens in Europe and the rest of the world and conforms to the aim of the FP7-HEALTH to contribute to international efforts addressing global health problems, such as antimicrobial drug resistance**.** One priority of the area of **antimicrobial drug resistance** within FP7-HEALTH is the discovery of new and urgently needed antibiotics.

The proposed work was organised into ten research work packages (WPs), one dissemination and exploitation WP and the project management WP. The research work included three major steps to identify novel drugs against multi-resistant *P. aeruginosa* and *B. cenocepacia*:

Target Identification (WP1, WP2, WP3)

applied a combination of advanced **genomic approaches** devised to screen the entire genome of *P. aeruginosa* for the identification of potential targets. Target identification was carried out using a combination of *P. aeruginosa* clinical epidemic and dominant strains including those resistant to antibiotics. The main molecular genetic approaches used in the identification of targets were:

- Improved **insertional mutagenesis** for the identification of genes involved in virulence and hence establishment and maintenance of *P. aeruginosa* infection.
- **Signature Tagged Mutagenesis** (STM) for the identification of genes expressed during infection and hence required for the adaptation of the pathogen to the host.
- Screening of **conditional antisense RNA** libraries for the identification of genes essential for growth.

The use of these three strategies was paramount and it provided a unique picture on the complexity of host/pathogen interaction of *P. aeruginosa* during infection and lead to the identification of the physiological role of the target gene(s).

Target Validation (WP4, WP5, WP6)

used a multifaceted approach, by a sequential cascade of models including **biofilm, human cell lines** wild type or carrying CFTR mutations, the nematode worm ***Caenorhabditis elegans***, ***Drosophila melanogaster*** and **mouse models** both for acute and chronic infection, either to provide the rationale and the proof-of-concept for the characterisation and validation of selected targets as candidates for the development of antibacterials.

This project also revealed the role in virulence of a significant number genes of unknown function in the genome of *P. aeruginosa* as well as invaluable insights into their conservation across different **clinical isolates** of this organism. Target validation was

carried out in a collection of *P. aeruginosa* clinical strains of different origins and searched in other gram negative bacteria such as, *B. cenocepacia* strains through **multiple genome sequencing** and **comparative analysis**. This provided a panel of targets with a global relevance and can be considered a critical step in terms of providing solid rationale to further proceed with the project towards the target inhibition.

Target Inhibition (WP7, WP8, WP9, WP10)

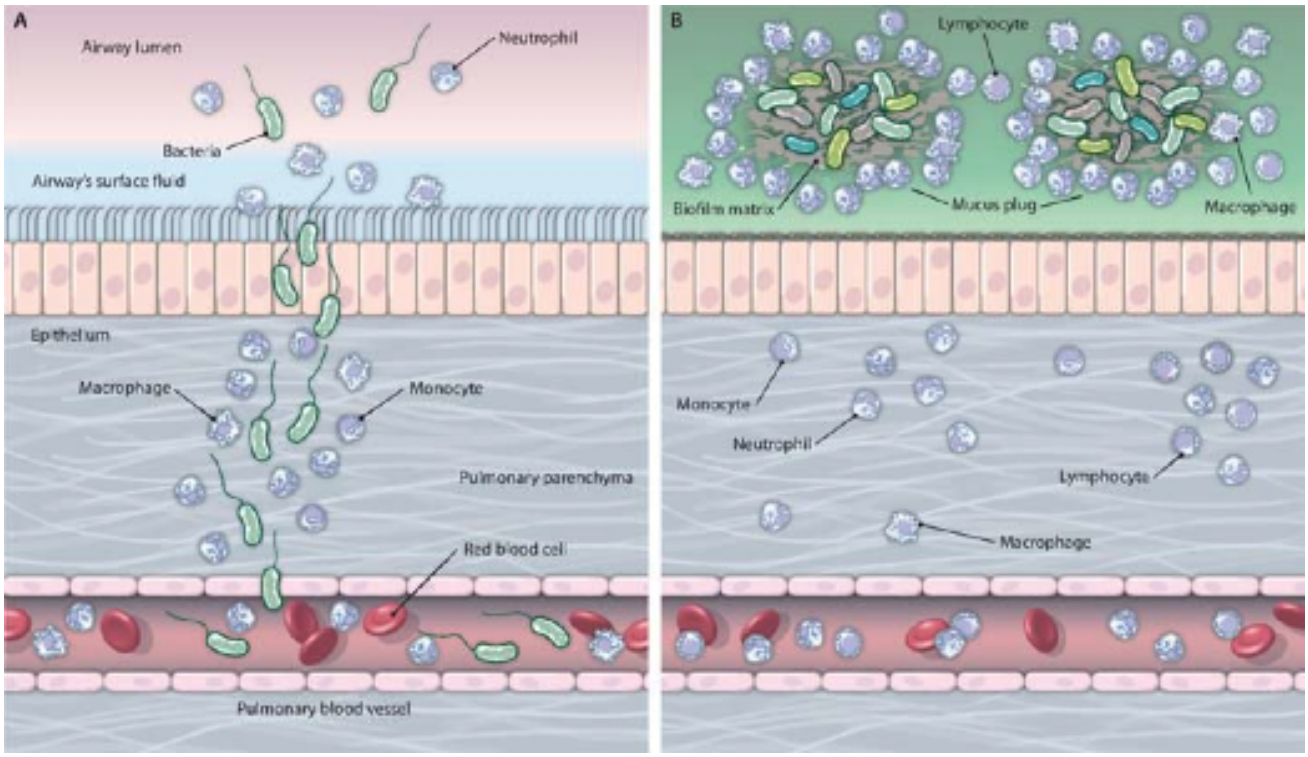
developed two alternative, yet complementary, strategies aimed to attack the problem of *P. aeruginosa* infection:

- novel drugs from the screening of chemical libraries against specifically selected targets, and the characterisation of the mode of action of novel drugs with antibacterial activity discovered by some members of the partnership.
- novel antisense inhibitors of bacterial targets by peptide nucleic acids (PNA) delivery.

The combined efforts resulted in a panel of new agents for the treatment of *P. aeruginosa* infections in patients with cystic fibrosis and other patient risk groups and a robust network of technologies that could also be applied for the development of anti-microbial drugs for other bacterial pathogens, especially other Gram-negative bacteria, such as *B. cenocepacia*.

WP1 - Search for novel virulence functions by the screening of transposon libraries.

The Gram-negative *P. aeruginosa* is a clinically relevant opportunistic pathogen highly resistant to most classes of antibiotics (Rehm, 2008). Both acute and chronic infections can be established in a wide range of patients. The conditions present in acute infections force pathogens to injure or kill the host, with multi-organ failure sometimes occurring in hours or days (A). In contrast, chronic infections occur without injury and in the presence of biofilm structures—a population of microorganisms that aggregates on a matrix—that develop over days or weeks, and bacterial genetic variants may grow in the biofilms (B) (Bragonzi, 2010a). *P. aeruginosa* virulence factors required for the initiation of acute infections are selected against during chronic infection. Thus, acute and chronic bacterial infections are different at cellular and organ levels and may require distinct treatments.

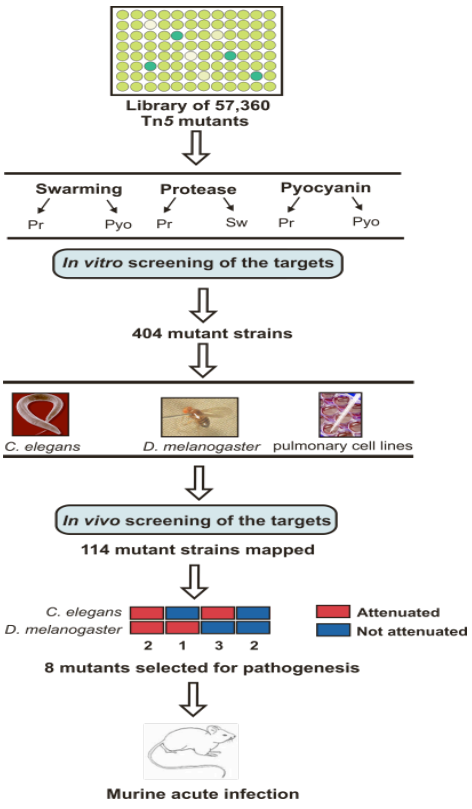


The genomes of *P. aeruginosa* strains are larger than those of most sequenced bacteria (Stover, 2000). The genome size varies between 6 to 7 Mbp with over 5.500 ORFs. A significant number (8.4%) of *P. aeruginosa* genes are predicted to be involved in regulation, which at the time of publication of the genome was the largest fraction of regulators among sequenced bacterial genomes. Around 32% of the genes in the *P. aeruginosa* PAO1 genome have no homology to any previous reported sequences and only 6.7% have experimentally demonstrated functions. This implies that there is likely a large number of genes to be discovered, that are involved in the virulence in this organism, and which will be coding for novel antimicrobial targets. These targets could be exploited for the development of novel therapies.

Two **genomic approaches** including functional genomics through insertion mutagenesis (**strategy A: acute virulence**) and signature-tagged mutagenesis (STM) technology

(**strategy B: chronic virulence**), has been used for the identification of novel targets in *P. aeruginosa* relevant in acute and chronic infection. In particular, the Tn5 mutagenesis approach used to generate transposon libraries is considered a powerful tool to examine a large number of mutants. Using this technology, it is possible to generate 60,000 mutants for each strain needed to cover a genome of 6.5 Mb (Liberati, 2006). The approach has identified novel genes involved in virulence and antibiotic resistance in microbial pathogens. An improved version of the Tn5 mutagenesis is the STM approach. STM uses transposons tagged with unique oligonucleotides for the simultaneous analysis of a large collection of mutants in vivo with a limited number of animals (Potvin, 2003). The approach has been used to identify novel *P. aeruginosa* genes involved in the adaptation of this pathogen to the host.

The **strategy A** was based on a multistep-driven screening for genes which mutation results in the **attenuation of virulence**. Using a **mutagenesis approach** by the transposon **Tn*5***, the University of Nottingham generated a total of 57,360 mutants in *P. aeruginosa* PAO1. The target screening was performed at different sequential levels. Firstly, mutants were individually tested for reduced swarming as well as pyocyanin and protease production. Secondly, a selection of those having pleiotropic phenotypes were further examined for attenuation in *Caenorhabditis elegans, Drosophila melanogaster* and, after a further shortlisting, for reduced cytotoxicity on respiratory cell lines (WP4). Finally, a total of 13 mutants resulting from these sequential screenings, including a selection of genes not attenuated in virulence used as controls, were verified as potential virulence targets using a combination of a mouse acute infection model, together with cell invasion assays and IL-8 release employing the A549 alveolar epithelial cell assay (see WP4) (manuscripts in preparation).



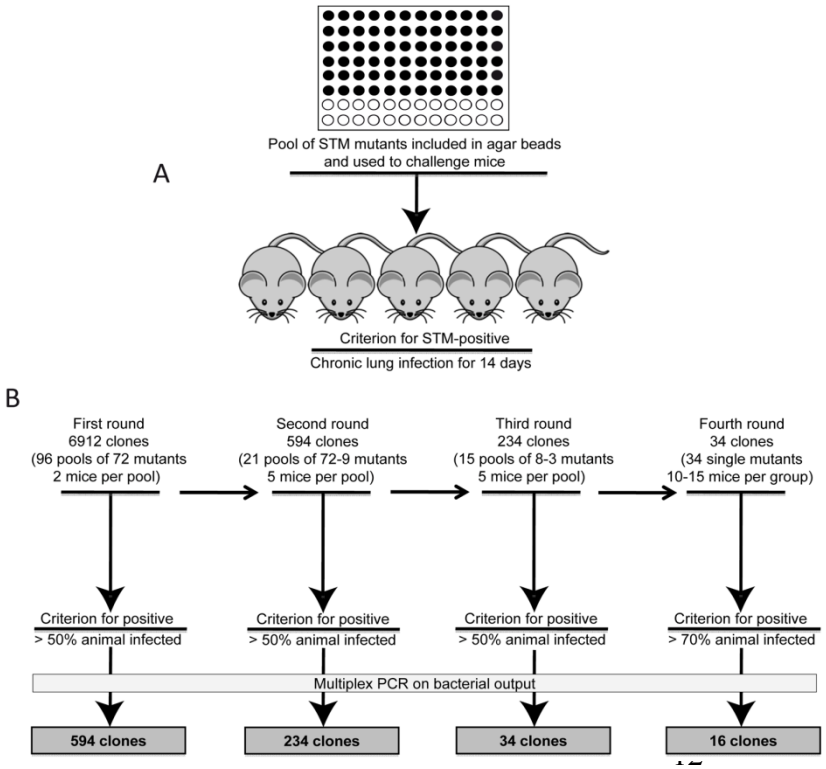
The screening of the transposon library resulted in the selection of 72 open reading frames (ORFs) with unique Tn5 insertions and attenuated in virulence. The predicted proteins encoded by these ORFs revealed that these genes are involved in various cellular functions spanning seventeen functional classes, including energy, amino acid and nucleotide metabolisms, transcriptional and post-transcriptional regulation, motility and adaptation, secretion and transport of small molecules, enzymes as well as conserved hypothetical proteins of unknown functions. There was a wide spread distribution for the predicted functional category of the mutated genes, with the highest abundance corresponding to 24 genes with unknown function followed at a distance by 7 genes involved in transport of small molecules. To address the global relevance of the target genes, a comparative protein sequence analysis was carried out against six sequenced genomes (PA14, LESB58, PA7, 2192, C3719 and PACS2) of highly pathogenic *P. aeruginosa* and against *E. coli* K12 and *B. cenocepacia* J2315 in order to check presence and conservation of our candidates in other *P. aeruginosa* genomes and

in other bacterial species (see WP5). The majority of the selected targets are conserved in all the six *P. aeruginosa* genomes analysed.

The mutants validated in the cell culture, *D. melanogaster* and *C. elegans* and animal model (see WP4) were analysed *in silico* in order to obtain information on their patentability, biochemical activity, 3-D structure (WP8), and conservation among clinical isolate (WP5). This resulted in the selection of **5 novel targets** to be further investigated for their pathogenicity. Since the use of Tn5 mutagenesis can result in the inactivation of more than one gene located in the vicinity of those mutated, it is important to make defined mutations in the specific genes identified in the screening to ensure that the correct target has been identified. The defined mutants were further validated in different model systems as described above. Results of this screening approach will be published soon.

The **strategy B** was based on the identification of genes involved in the **adaptation of *P. aeruginosa* to the host**. A novel STM positive (**Pos-STM**) approach in *P. aeruginosa* was reported for the first time by Università Vita e Salute (Bianconi, 2011). The latter study was undertaken to screen for bacterial maintenance, as opposed to elimination (Potvin, 2003). The study was undertaken on the premise that loss-of function mutations in *P. aeruginosa* isolates from CF patients enhance fitness and sustain chronic infection.

A novel STM screening based on positive selection by using *P. aeruginosa* PAO1 STM library as source of mutants (Potvin, 2003) and the validated agar beads mouse model of *P. aeruginosa* chronic infection was designed and published in PLoS Pathogen (Bianconi, 2011). The source of mutants was a *P. aeruginosa* PAO1 STM mini-*Tn5* library constructed previously (Potvin, 2003). Thus, Pos-STM screening of a collection of 7968 *P. aeruginosa* PAO1 mutants in a murine model of chronic airway infection identified 16 mutants. The mini-Tn5 insertion of each Pos-STM mutant was mapped by sequencing. The 15 insertion sites mapped within a specific *P. aeruginosa* gene while the remaining one was mapped in an intergenic region (PA0436-PA0437). Interestingly, the previous report showed that eight different intergenic regions were mutated in the 96-month isolate from CF patients (Smith, 2006). The 15 mini-Tn5 inserted genes encoded proteins from almost all functional classes: hypothetical, unknown, unclassified proteins (PA2972, PA4842, PA5028), motility and attachment (PA0410-*pilI,* PA0499, PA1077-*flgB,* PA4554-*pilY1*), putative enzymes (PA1856), transport of small molecules (PA0890-*aotM,* PA2252, PA4887), amino acid biosynthesis and metabolism (PA0895-*aruC*), energy metabolism (PA2998-*nqrB*), secreted factors (PA3478-*rhlB*), chaperones and heat shock proteins (PA5053-*hslV*). The phenotypes associated to these

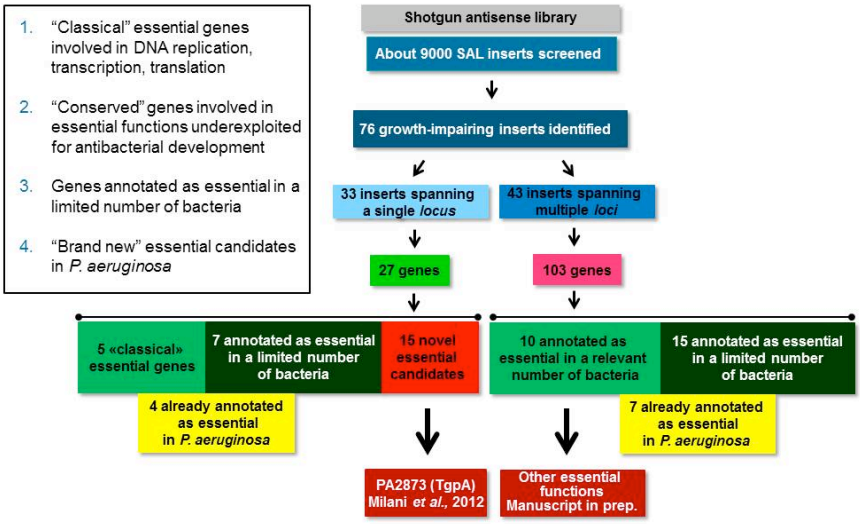


Pos-STM mutations reflect alterations in diverse aspects of *P. aeruginosa* biology which include lack of swimming and twitching motility, lack of production of the virulence factors such as pyocyanin, biofilm formation, and metabolic functions. *RhlB* and *pilY1* specific *loci* were identified independently by both Pos-STM and Neg-STM approach, supporting previous hypothesis in *P. aeruginosa* that virulence factors essential for acute infection are lost when *P. aeruginosa* establishes long-term chronic infection. The reliability and robustness of Pos-STM approach is supported by the high proportion of mutations found in Pos-STM genes of longitudinally *P. aeruginosa* isolates from CF patients as verified in WP5. This approach generated a list of genes whose inactivation increased the colonisation and persistence in chronic airways infection in murine model similarly to clinical strains isolated from patients with CF.

Animal studies were carried out in strict accordance with the Ministry of Health guidelines for the use and care of experimental animals. This study was approved by the San Raffaele Scientific Institute (Italy), University of Tuebingen (Germany) and University of Notthingam (UK) Institutional Animal Care and Use Committee (IACUC). All efforts were made to minimize the number of animals used and their suffering.

WP2 - Novel essential functions by the screening of antisense libraries

Along with virulence functions (WP1), NABATIVI aimed at identifying novel targets of *P. aeruginosa* with an essential role for its survival. For this specific purpose, University of Milan adopted in WP2 a genetic technique called "**shotgun antisense screening**" that was developed in the Gram-positive bacterium *Staphylococcus aureus* and, following a period of limited success in Gram-negative bacteria (like *P. aeruginosa*), has recently been used effectively in the well-known model bacterium *Escherichia coli*. To further improve the method and also target low expressed essential genes, we included in our protocol some variant steps that were expected to overcome the non-stringent regulation of the promoter carried by the expression vector used for the shotgun antisense libraries. Our antisense screenings identified 33 growth-impairing single-locus genomic inserts that allowed us to generate a list of 27 "essential-for-growth" genes: five were "classical" essential genes involved in DNA replication, transcription, translation, and cell division; seven were already reported as essential in other bacteria; and 15 were "novel" essential genes with no homologs reported to have an essential role in other bacterial species. Interestingly, the essential genes in our panel were suggested to take part in a broader range of cellular functions than those currently targeted by extant antibiotics, namely protein secretion, biosynthesis of cofactors, prosthetic groups and carriers, energy metabolism, central intermediary metabolism, transport of small molecules, translation, post-translational modification, non-ribosomal peptide synthesis, lipopolysaccharide synthesis/modification, and transcription regulation.

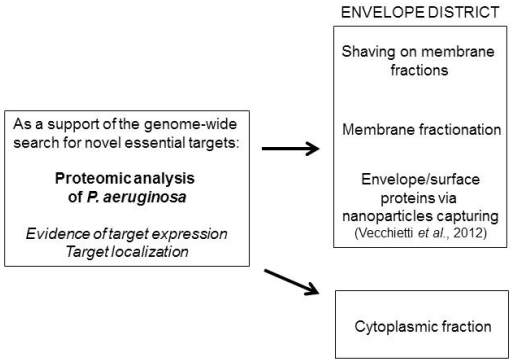


This study also identified 43 growth-impairing inserts carrying multiple loci targeting 105 genes, of which 25 have homologs reported as essential in other bacteria.

Taken together, our results show the feasibility of antisense technology in *P. aeruginosa* for identifying novel essential genes. Because of its supposed inefficiency, this approach has been neglected in Gram-negative bacteria for several years, and was only recently recovered in *E. coli*. By comparison with this previous work, the WP2 results strongly suggest that our modification of the antisense strategy can broaden the class variety of the identified essential genes. We expect that our methodology could be well suited for antisense-mediated searches of essential genes in other Gram-negative bacterial species. The results of our antisense screenings will be published soon and become available as source of novel antibacterial targets for *P. aeruginosa.*

WP2 also aimed at the characterization of novel essential functions in *P. aeruginosa*. For this purpose, we considered hits that were found by the antisense screenings. In particular, we focused on the **PA2873** gene product that was annotated as a hypothetical membrane protein endowed with a periplasmic region harbouring a structural domain belonging to the transglutaminase-like superfamily, a group of archaeal, bacterial and eukaryotic proteins homologous to animal transglutaminases. In the study recently published in PLoS ONE (Milani, 2012), University of Milano shows that the periplasmic portion of the PA2873 protein, which we named **TgpA**, does possess transglutaminase activity *in vitro*. This is the first report of transglutaminase activity in *P. aeruginosa*. In addition, we have provided strong evidences that TgpA plays a critical role in the viability of *P. aeruginosa* and is a good candidate as antibacterial target. The characterization of other novel essential functions from the "antisense panel" is in progress.

For the refinement of raw data, a genome-wide approach, as was our antisense screening, necessarily relies on the information about protein function and localization present in the genomic database. However, it is very common that in the sequenced bacterial genomes the percentage of proteins annotated as "hypothetical" (i.e. only predicted *in silico* and no experimental evidence of *in vivo* expression) is very high. Likewise, very frequently the subcellular localization confidence is based on pure algorithms. Therefore, as a support of the genome-wide approach of this and other WPs (e.g. WP1), we performed a multiview proteomic analysis of *P. aeruginosa* to get hints about target expression and localization. In this context, University of Milano developed an innovative proteomic technique for the analysis of the envelope district (Vecchietti, 2012)



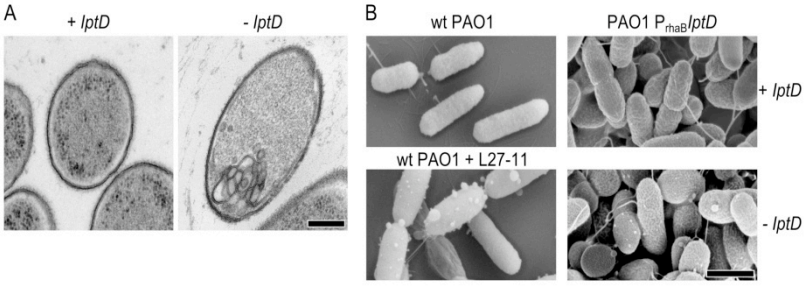
Finally, as antibacterial targets, we did not focus only on proteins but also on small RNAs (sRNAs), which several studies suggest to play key roles in regulating cellular processes linked to pathogenesis and essential functions. The number of such regulatory molecules previously identified and annotated in *P. aeruginosa* was relatively low, considering its genome size, phenotypic diversity and adaptability. The apparent low proportion of sRNAs in *P. aeruginosa* could reflect either a real paucity of regulatory sRNAs or the limited number of genome-wide searches that have been performed in this species. Therefore, we accomplished a deep-sequencing approach to explore the *P. aeruginosa* complement of sRNAs (Ferrara, 2012).

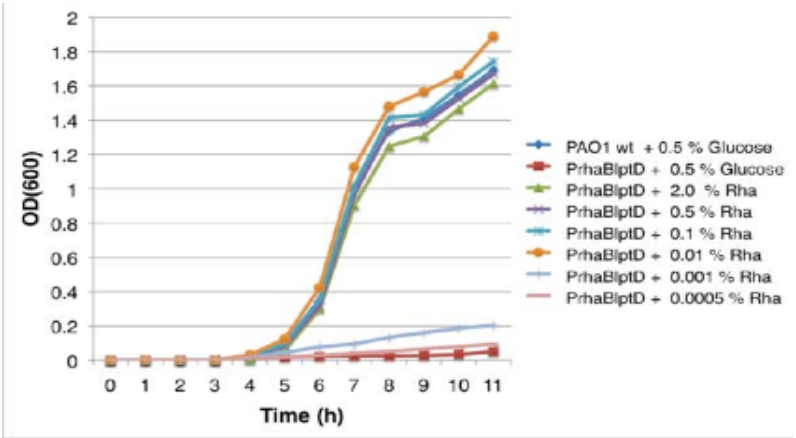
WP3 - Identification and characterisation of targets inhibited by peptidomimetic antimicrobials

At the time of this application, a novel compound class derived from **natural antimicrobial peptide protegrin I** was discovered as potent antibiotic against Gram-negative *P. aeruginosa* by University of Zürich and Polyphor Ltd (Srinivas, 2010). The target (gene) and mode of action of **novel peptidomimetic antimicrobials** was elucidated during the drug discovery process within the NABATIVI project.

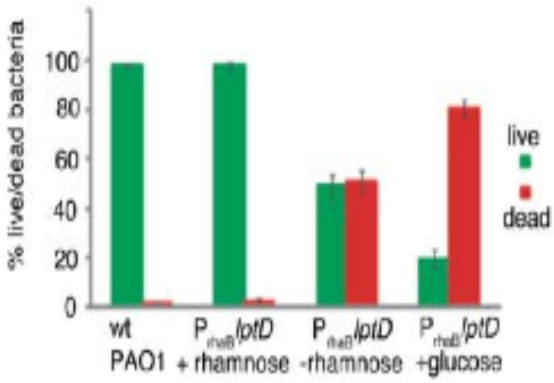
Photoaffinity labelling experiments were designed to test the hypothesis that the peptidomimetic antibiotics bind to the outer membrane (OM) protein **LptD** in *P. aeruginosa* PAO1. A petidomimetic with a photoaffinity labelling reagent (**PAL-1**, with an MIC against *PA* PAO1 of 0.05 µg/ml) was designed for this purpose. Photoaffinity labelling with **PAL-1** consistently revealed one major photo-labeled protein, confirmed to be LptD (**PA0595**) by in-gel protease digestion/LC-ESI-MS-MS analysis and Western blot. When the photoaffinity labelling was repeated in the presence of a 100x excess of the antibiotic (**L27-11**), the labelled band disappeared from the blot, demonstrating a direct competition between **L27-11** and **PAL-1** for binding to the target. Furthermore, when the experiment was repeated using the resistant PAO1^{RES1} mutant (containing a mutated *lptD* gene), photolabelling of LptD was not detected, indicating that the mutated LptD protein is no longer able to bind the antibiotic with comparable affinity. These results provide firm support for the conclusion that the peptidomimetic antibiotics bind with high specificity to LptD in the OM of intact *PA* cells.

Electron microscopy studies were carried out in an effort to detect morphological changes to cells caused by the antibiotics. For this purpose, *P. aeruginosa* PAO1 cells grown for 3-5 h in the presence of antibiotic were examined in thin sections by transmission electron microscopy (EM). After fixation many apparently intact cells showed remarkable accumulations internally of membrane-like material, an effect not seen in cells grown normally without the antibiotic, nor in cells of the resistant PAO1^{RES1} mutant grown in the presence of the antibiotic. Similar accumulations of extra membranous material were reported in *E. coli* cells depleted of *lptD*, and in other bacteria exposed to antimicrobial peptides. These results provide a first indication that the peptidomimetics have an impact on outer membrane biogenesis, resulting in the appearance of a membrane-stress-like response.



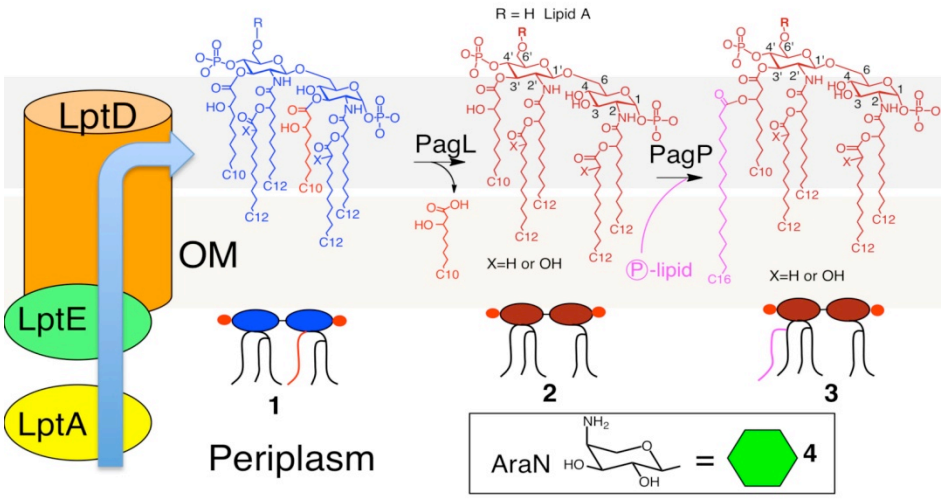


To confirm the essentiality of *ostA*, a conditional mutant was constructed and grown in the



presence of either glucose or rhamnose. *ostA* mutant grew in the presence of 0.5 % rhamnose similarly to the wild type but was unable to grow in 0.5 % glucose on both plates and liquid media as expected for conditional mutant with an essential gene under the control of the rhamnose promoter. Depletion experiment using liquid media also showed that the *ostA* mutant was unable to grow after subculturing into fresh medium supplemented with glucose instead of rhamnose. To further confirm that *ostA* is essential for the viability, the cells at the 11 hour time point of the growth were stained with the Baclight Live/Dead stain and examined by the phase contrast and fluorescent microscopy. When grown under inducting conditions (0.005 % rhamnose) the *ostA* mutant cells were green/alive similar to the wild type, while under inhibiting conditions (0.005 % glucose and 0.001 % rhamnose) the majority of the *ostA* mutant cells were red/dead, thus proving that *ostA* is important not only for growth but also for the survival of *Pseudomonas*.

Down-regulation of *lptD* expression should produce effects on lipid A modification that are similar to those induced by the antibiotic. To test this, lipid A was prepared from the conditional PAO1 mutant grown under permissive conditions and analysed by ESI-MS. The MS spectrum showed mostly the same lipid A species as seen from the wt PAO1 (coloured red below). Under limiting growth conditions, however, bacterial growth was slower and the extracted lipid A showed accumulation of the same lipid A species seen when the wt strain is exposed to antibiotic (blue lipid A). Thus, down-regulation of lptD leads to the accumulation of LPS forms that have not been processed by PagL, just like in the case of antibiotic-treated cells.



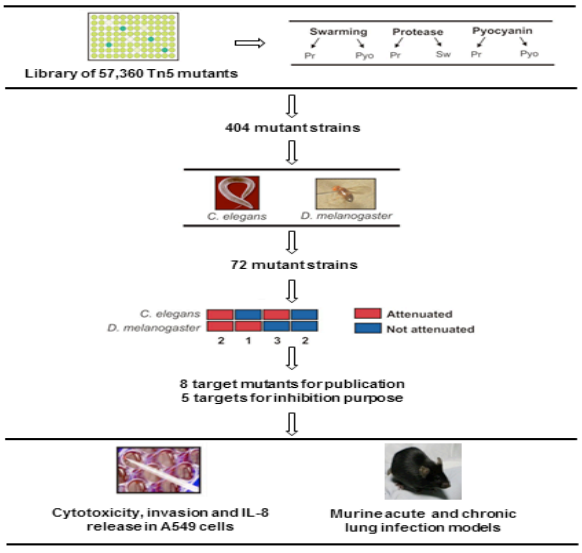
In summary, we have gained substantial new evidence that the peptidomimetic antibiotics exert their antimicrobial effect by interacting with LptD and **inhibiting the transport of LPS to the cell surface**. This is the first example of antibiotics targeting LptD and having a new mode of action, representing a valuable tool to combat emerging resistance to the currently used treatments. The results have been published in Science (Srinivas, 2010) and commented in Science Translational Medicine (Bragonzi, 2010).

WP4 - Characterization of the novel targets by a cascade of different model systems

The identification of targets carried out mainly *in vitro* by genome-wide screenings (see WP1-2) does not imply that they are relevant for their pathogenesis *in vivo*. A pathogen can experience several radically different "host environments" at various stages of infection (acute or chronic). In the context of infections caused by *P. aeruginosa* virulence is mediated by a wide variety of trans-acting regulators that sense the environment and the physiological state of the cell and adjust the transcription of specific genes changing their phenotype significantly. The idea of a core set of virulence factors common to all infection models from plants and insects to humans is thus unlikely. Therefore, we need a multifaceted approach, by using a **sequential cascade of models** to provide the rationale and the proof-of-concept for the characterisation and validation of a selected bacterial target as candidate for the development of antibacterials.

To address this question we have used a new **validation strategy** based on a cascade of *in vitro* and *in vivo* virulence models including **airways cells**, ***C. elegans***, ***D. melanogaster*** and **mouse models** of acute and chronic lung infection. In WP1, the University of Nottingham constructed a Tn*5* mutant library in *P. aeruginosa* PAO1. Among a total of 57,360 mutant strains, 404 mutants were found to be attenuated in virulence factors including swarming, pyocyanin and protease production. These mutants showing pleiotropic phenotypes were tested in a second stage screening by the University of Zurich. This was based on the use of *C. elegans* and *D. melanogaster* as infection hosts to identify attenuated mutants. The aim was to further shortlist these mutants prior to evaluate their cytotoxicity and virulence in eukaryotic cells lines and murine models. Indeed, although rodents are the first choice for understanding infectious diseases in human, screening a large amount of targets in mouse models is unfeasible.

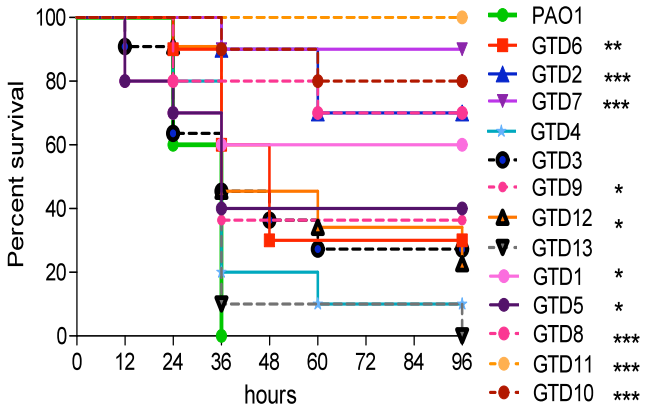
From a total of 404 transposon mutants tested, 108 were found to be attenuated in at least one of the infection models. This screening and the identification of the insertion sites to eliminate mutants redundancy reduced the numbers of mutants with attenuated virulence in at least one non-mammalian model to 72 target genes. A **Genomic Target Database** (**GTD**) has been developed by scoring the target genes for virulence traits, phenotype and pathogenicity in different model systems. Next, we selected **five mutant** strains attenuated in all the models to be exploited for the inhibition stage of the NABATIVI project (see WP8). All these five mutants have an **unknown functions** and are potentially **patentable**. In addition, eight mutant strains attenuated in one, both or none of the *C. elegans* and *D. melanogaster* disease models for publication purpose. These mutants show a **proof of concept** of the **validation strategy**.



Stable ko specific mutants were generated (see WP1) and further validation tests included: (i) additional phenotyping including biofilm formation, ability to produce elastase and pyoverdine, (ii) *C. elegans* and *D. melanogaster* killing curves, (iii) cell

cytotoxicity, (iv) invasion and (v) cytokine production assays. All the 13 selected mutants showed a reduction in surface motility, biofilm formation, and their ability to produce elastase and pyoverdine. The impact of the total 13 selected mutations on *D. melanogaster* and *C. elegans* survival was confirmed for the majority of the target genes repeating the infections with stable ko mutants. In accordance with the previous outcomes of the sequential screening cascade approach Eberhard Karls Universitat and Università Vita-Salute San Raffaele challenged A549 alveolar epithelial cells with the mutants in order to validate the attenuation of the candidates in terms of cytotoxicity, invasion capacity and secretion of the pro-inflammatory cytokine IL-8. Six out of the eight mutants selected for publication and three of five mutants selected for inhibition purpose were attenuated in their virulence in immortalized cells.

Finally, Università Vita-Salute San Raffaele tested *P. aeruginosa* selected mutants for lethality in the mammalian host using a murine model of acute lung infection, by intra-tracheal injection of planktonic bacterial cells. While wild type PAO1 was totally lethal within 36h, the lethality of some mutants was significantly lower and temporally shifted. Five out of eight mutants for publication purpose and two out of five mutants chosen for the inhibition stage were significantly attenuated in inducing mortality when compared to wild type PAO1-L. It should be noticed that some mutants attenuated in *C. elegans* were not attenuated in the mouse model. Our data show that identification of virulence genes carried out mainly *in vitro* does not imply that they are relevant for their pathogenesis *in vivo*, suggesting a host-specific response to *P. aeruginosa* and indicating the necessity to test a selection of mutants in the mouse model, as the nearest to the human host.



Acute *P. aeruginosa* infection in immuno-compromised patients or chronic persistent lung infection in patients with CF are different at the cellular and organ levels and thus may require distinct treatments. To mimic the environmental conditions present in the lung of CF patients, a mouse model of chronic lung infection, by intra-tracheal injection of agar-bead encased bacteria, was established in mice by Eberhard Karls Universitat and Università Vita-Salute San Raffaele (Bragonzi, 2010b). Five targets selected for the inhibition step were tested. Results showed that one of the target mutants, attenuated in all the previous screenings, was highly reduced in lethality in the murine model of chronic infection, thus representing the most promising candidate for drug development. In addition these results confirm that a **multifaceted bottleneck approach**, by using different screening systems to provide the rationale and the proof-of-concept, is essential for the characterization and validation of a large number of bacterial target candidates.

In summary, by using a genomic approach devised to screen the entire *P. aeruginosa* genome for novel virulence genes (WP1) and a multifaceted approach (WP4) based on a sequential cascade of models for the validation step, we have identified several novel virulence genes. These targets have been further investigated for their function and for their inhibition (WP7), representing interesting targets for innovative anti-virulence approach to *P. aeruginosa* infections.

WP5 - Validation of the targets in strains of various clinical origin

One limitation of many identified targets described during the past three decades is that the identified targets showed sequence variability, and although they induced protection against homologous strains, they failed to induce protection against heterologous ones. **Clinical relevance** means that the new targets should be adequate to cover a spectrum of pathogens faced by a physician, thus providing the opportunity for the generation of new antibacterials. To address this need, NABATIVI exploited genomic sequences of *P. aeruginosa* strains from different origins and other gram-negative bacteria and use this information to select validated targets as clinically relevant. Thus, the aim of this work package was to validate the targets selected during the project in strains of various clinical origin by multiple **genome sequence** and **comparative analysis**.

The genome-wide screenings for the putative sensitive bacterial targets in NABATIVI has been carried out mainly on the laboratory reference strain *P. aeruginosa* PAO1, and at some extent on *P. aeruginosa* dominant strain PA14. As first step, to determine the relevance of our panel as therapeutic targets, Università Vita-Salute San Raffaele carried out comparative analysis in order to check whether 226 targets selected in WP1, WP2 and WP3 were present in six sequenced genomes of *P. aeruginosa* (PA14, LESB58, PA7, 2192, C3719 and PACS2). For homology analysis the BLASTP program present on the NCBI website was used. The analysis was done by aligning the amino acid sequence of PAO1 against the other six genomes of *P. aeruginosa*. We generated a target database containing information about the class of confidence and the functional classification of the proteins, the strain in which the target has been identified and the percentage of identity of the protein in each strain compared to PAO1. The database of targets has been grouped by ranking the following scores:

- the degree of conservation;
- the presence in all six sequenced strains;
- the presence of ortholog genes in the same strain;
- the target confidence class.

Although the screening for novel targets was carried out in *P. aeruginosa* as model bacterium for studying opportunistic pathogens, any identified targets has been searched and validated in other gram-negative bacteria, such as *Burkholderia cenocepacia* and *Escherichia coli*. Some *Burkholderia* strains and species have been determined to be transmissible between patients either in hospitals or clinics or through contact between CF patients outside clinical settings. *E. coli* is known as one of the most common pathogens that cause nosocomial infections, together with *P. aeruginosa* and *Staphylococcus aureus*. A comparative genome analysis of the selected targets was performed in *B. cenocepacia* J2315 and *E. coli* K12 strains with the goal to identify all the target that are common or specific for one or the other strain, therefore providing an overall complete list of universal encoded potential targets. Furthermore, the targets were also analysed to verify the absence of similar proteins in humans.

All the results obtained in this WP and in WP4 were used to generate a **genomic target database** (**GTD**) of 72 selected targets. An exemplificative picture of this database is shown below. Sequences analysis of virulence genes identified by insertional mutagenesis and

validated by a sequential different models outlined in WP4 showed that these **genes** were **conserved** and not mutated thus representing excellent candidate targets for inhibition.

Genome Target Database

GTD number	Class of confidence	In silico analysis			*In vitro* phenotypic analysis			Non mammalian models		Screening in immortalized cells				Murine model
		Homology (range) to all sequenced *P. aeruginosa* genomes*	Homology to *B. cenocepacia* J2315	Homology to human genome	Protease production	Swarming motility	Pyocyanin production	*C. elegans*	*D. melanogaster*	Bacteria cytotoxicity in A549	SN cytotoxicity in A549	Invasion In A549	IL-8 production in A549	Acute murine lung infection
GTD 3	2	94-99%	25%	NSH	-	-	-	-	-	-	-	-	-	+
GTD 4	2	98-100%	60%	28%	-	-	-	-	-	-	-	-	-	+
GTD 1	4	92-99%	38%	NSH	-	-	-	-	-	-	+	-	-	-
GTD 5	4	99-100%	65%	NSH	-	-	-	-	+	+	-	-	-	-
GTD 10	4	33-100%	NSH	22%	-	-	-	-	-	-	-	-	-	-

**P. aeruginosa* sequenced genomes (LESB58, PA14, PA7, 2192, C3719, PACS2)
NSH not significant homology
- attenuated in comparison to wild type PAO1
+ not attenuated in comparison to wild type PAO1

It has been recently shown that bacterial functions needed for acute and generalizing types of infections, such as those occurring in immunosuppressed patients are selected against during the progression toward localised chronic infections (Smith, 2006). Indeed, many targets that would be considered for antibacterial development, may be expressed at low levels or absent from the majority of isolates during chronic infection. While these targets may be useful to eliminate the initially infecting strains, it is not clear whether their inhibition will control or promote progression towards the chronic infection. Thus, conservation of targets in phenotypically and genotypically different *P. aeruginosa* strains generated during the course of chronic infection, is required to develop an effective antimicrobial against *P. aeruginosa*. To address this issue, Università Vita-Salute San Raffaele evaluated the panel of targets, selected during the course of NABATIVI project, in terms of genetic diversity and frequency of recombination. This was intended to provide clinical validation of selected targets and solid rationale to further proceed with the project towards the targets inhibition. To this end, twenty-five sequential *P. aeruginosa* isolates from 6 patients with CF were chosen from the strains collection of the Hannover CF center (Germany), including early strains isolated at the onset of chronic colonisation and late clonal strains collected over a period of up to 16.3 years. In addition, the collection was implemented with strains isolated from respiratory specimens of 345 CF patients recruited in one of the largest centre in Europe (Cystic Fibrosis Center of Verona, Italy) for an epidemiological study aimed to establish the potential presence of epidemic strains. Phenotypic and genotypic analysis have been carried out in the strains of these collections. In details, the isolates were characterised for the presence of specific phenotypic traits associated with the acute phases of infection (twitching and swimming motility, production of proteases, siderophore and pyocyanin, haemolysis) or chronic phases of infection (mucoidy, LasR).

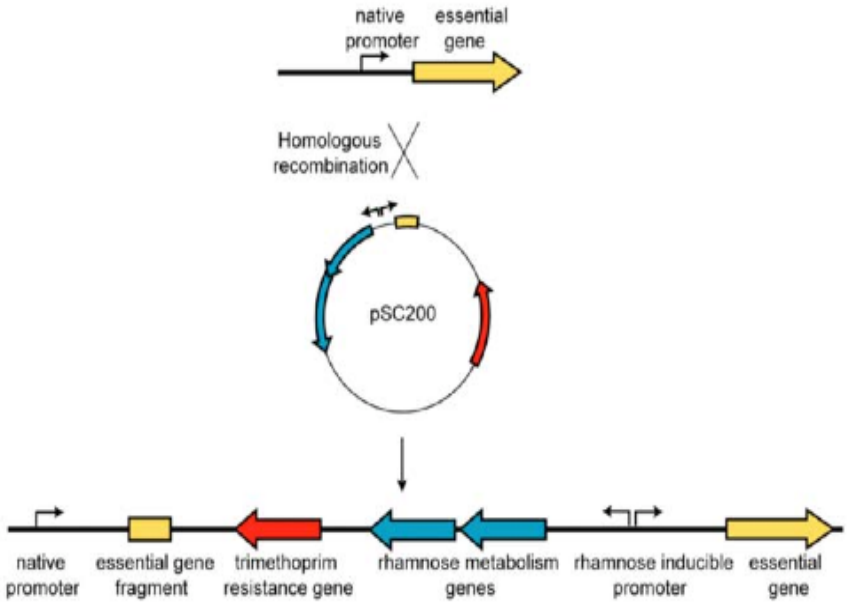
To address the issue of **target conservation**, Università Vita-Salute San Raffaele set out to sequence 16 target genes which were selected by **Pos-STM** (see WP1). Sequences analysis of the genes by comparison between early and late strains revealed single base-pair synonymous and non-synonymous mutations in seven of 16 genes in six different clonal lineages. Non-synonymous mutations were found in five genes from five different clonal lineages of CF patients**.** A standard computational method for predicting the effect of each **non-synonymous mutations** on protein function suggested that the non-tolerated changes in three genes are likely to affect protein function. This approach generated a list of genes whose inactivation increased the colonisation and persistence in chronic airways infection.

WP6 - Exploitation of known targets

Previous studies performed by NABATIVI participants have revealed the existence of a number of potential antibacterial targets in *P. aeruginosa* and *B. cenocepacia.* Amongst these are **quorum sensing** (QS) systems which comprise highly attractive novel therapeutic targets, which have the potential to substitute or complement traditional antibiotic treatments of chronic diseases (Hentzer, 2003). The QS response orchestrates the expression of a cocktail of virulence factors in a population density-dependent manner in *P. aeruginosa* as well as in *B. cenocepacia*. Therefore blocking of the QS cascades in these Gram-negative bacteria is a very promising approach to attenuate their pathogenicity.

The aim of this work package was to develop **bioreporter systems** using known and new targets which can be exploited for **high throughput screening** (HTS). Initial work focused on the exploitation of QS-related targets in Gram-negative bacteria, particularly the *N*-acyl homoserine lactone (AHL)-dependent QS system operating in *B. cenocepacia* and the *Pseudomonas* quinolone signal (PQS)- dependent QS system of *P. aeruginosa.*

To develop a platform for the screening of compounds interfering with PQS signalling in *P. aeruginosa* a biosensors was constructed by University of Nottingham utilising promoter regions of ***lasI***, ***lasR***, ***rhlI***, ***rhlR***, ***pqsA*** and ***pqsR*****.** The biosensors were sent to Actar-KDevExploratory to screen for compounds, which could potentially block the synthesis of QS signal molecules (see WP7). For the screening of compounds interfering with octanoyl-homoserine lactone (C8-HSL)-dependent QS in *B. cenocepacia* biosensors suitable for mass screen of compound libraries were constructed by University of Zurich utilizing GFP-based biosensors in different genetic backgrounds. The resulting strains were then characterized with respect to their potential to screen for compounds interfering with QS in *B. cenocepacia*.

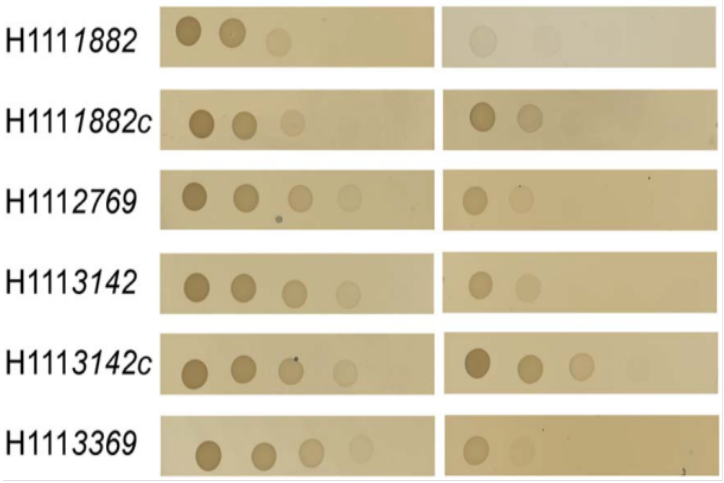


Generation of conditional mutants. Conditional mutants used to investigate essentiality of selected *Burkholderia* genes were generated by replacement of the candidate essential genes native promoters for the rhamnose-inducible promoter.

Another task in this work package was the construction of **whole cell reporter screens** for compounds that inhibit **OstA** of *P. aeruginosa*, the target of compound POL7001, which is investigated by Polyphor Ltd. To this end a conditional *ostA* mutant, in which the natural *ostA* promoter has been exchanged for a rhamnose-inducible one, was constructed. Growth experiments in which a culture of this strain is shifted to a

medium lacking arabinose unequivocally demonstrated an essential role of *ostA* for cell viability (see WP3 for details). We have performed a transcriptome analysis of the conditional mutant under permissive and non-permissive conditions and identified differentially regulated genes when *ostA* expression is abolished or the wild type strain is treated with POL7001. These promoters will be valuable for the construction of biosensors to screen and further analyse OstA inhibitors.

A third aim of this work package was the identification of novel **antibacterial targets** in ***B. cenocepacia***. Employing a bioinformatics approach we determined that the core genome of the order *Burkholderiales* consists of 649 genes. All but two of these identified genes were located on chromosome 1 of *B. cenocepacia*. Although many of these 649 core genes have been shown to be essential in other bacteria, we were also able to identify a number of novel essential genes present mainly, or exclusively, within this order. To test the essentiality of the identified genes conditional knock-down mutants in which the native promoter of the target gene or operon has been exchanged with a rhamnose-inducible promoter (as described above for OstA) were constructed. In this approach, approximately 300 bp fragments spanning the 5' region of a targeted gene were cloned into pSC200 and the resulting recombinant plasmids were subsequently transferred into the model strain *B. cenocepacia* H111 by triparental mating. Essentiality of target genes was examined by growing the strains in medium containing either rhamnose (permissive) or glucose (non-permissive). All conditional mutants grew well in the presence of rhamnose but were unable to grow in the presence of glucose on agar plates or in liquid medium. Using this approach the essentiality of some of the core genes, including the known essential genes *infB*, *gyrB*, *ubiB*, and *valS*, as well as the so far uncharacterized genes *BCAL1882*, *BCAL2769*, *BCAL3142* and *BCAL3369* could be confirmed experimentally. These latter genes have not previously been shown to be essential and thus represent **novel targets** for the development of **anti-*Burkholderia* drugs**.



NABATIVI has identified a handful of top targets which are currently under patent consideration. One of the approaches used was to identify compounds which could prevent the production of these targets and hence attenuate the virulence of *P. aeruginosa*. For this purpose, the University of Nottingham generated a collection of biosensors based on the construction of CTX *lux*-based chromosomal fusions to the promoter of each of these targets so that their expression could be monitored through the production of light. This was done to enable the identification of compounds which could switch off the expression of the genes coding for these targets using a high throughput approach. These biosensors also enabled the monitoring of the expression of these targets through growth.

WP7 - *Screening of natural and chemical compound libraries and lead compound generation*

The objectives of this WP were to develop appropriate **HTS assays** and then screen collections of **natural and synthetic compound libraries** to select **inhibitors** of targets identified and validated by partners in NABATIVI consortium. The WP7 output was a wide panel of small molecules, both hit and lead compounds, expected to be further optimized and well-suited to enter a portfolio of candidates for pre-clinical trials.

WP7 was successfully completed with vHTS and HTS assays on known target from QS (see WP6) and 4 novel **virulence** targets selected, identified and validated by NABATIVI consortium in WP1. In addition, HTS assays were developed and applied to select inhibitors of two **essential** targets identified and validated by NABATIVI consortium in WP2. Additional screening have been initiated of natural compound and plant extract libraries for growth inhibition activity on *P. aeruginosa* PAO1 to enhance the possibility to identify hits endowed with antibiotic potential.

HTS bioluminescence assays were developed for screening of **QS inhibitors** based on *P. aeruginosa*. 12,500 synthetic compounds, 480 natural purified compounds and 800 plant extracts of libraries were screened on PA14 biosensors, PA14 and PAO1. Using structural data on targets of interest, 25,000 original synthetic compounds from Actar-KDevExploratory, 20 million available products from Ambinter.com and 150,000 natural compound structures from Greenpharma were screened with in silico methods (WP8). Starting with 597 actives anti-QS synthetic and 13 natural anti-QS compounds, finally 21 synthetic compounds and 8 natural compounds were confirmed as anti-QS hits. 8 scaffolds from anti-QS screening were selected with good potential in IP and freedom to operate in SAR. 42 extracts reduced QS expression, which activity guided fractionation is still under way. 7 natural compounds and 8 extracts with growth inhibiting activity on PAO1 strain were identified. The scaffolds were optimized and champion compounds were tested in available phenotypic assays, by LCMS to identified direct effect on QS signal molecule production.

Comp	pqsA	lasI	rhlI	lasB	rhlA	pKan	Pyocyanin	Elastase	Protease	C4-HSL	OC12-HSL	HHQ	PQS
8D2	66	73	70	88	85	84	66	65	61	37	29	50	47
16F2	49	34	25	46	18	86	59	62	55	58	48	61	54
23E7	77	93	85	103	114	85	90	82	87	81	79	103	67
28G4	78	88	88	106	114	77	83	73	87	88	74	96	84
28G11	28	49	37	24	32	97	29	32	28	37	4	12	33
34E5	8	21	36	18	19	86	12	30	21	16	4	19	21
51D4	62	77	92	83	78	78	71	79	71	69	74	91	73
68A4	48	68	102	101	77	76	56	68	57	72	66	72	42
79H5	17	24	33	30	27	84	5	29	21	13	2	9	15

Example of results from compound screening. Results expressed as remaining activity of compounds tested for inhibition of QS genes, QS molecule levels and virulence factors controlled by QS.

Four novel targets from *P. aeruginosa* GTD identified and validated by NABATIVI consortium were proceed in screening assay development. HTS biosensor

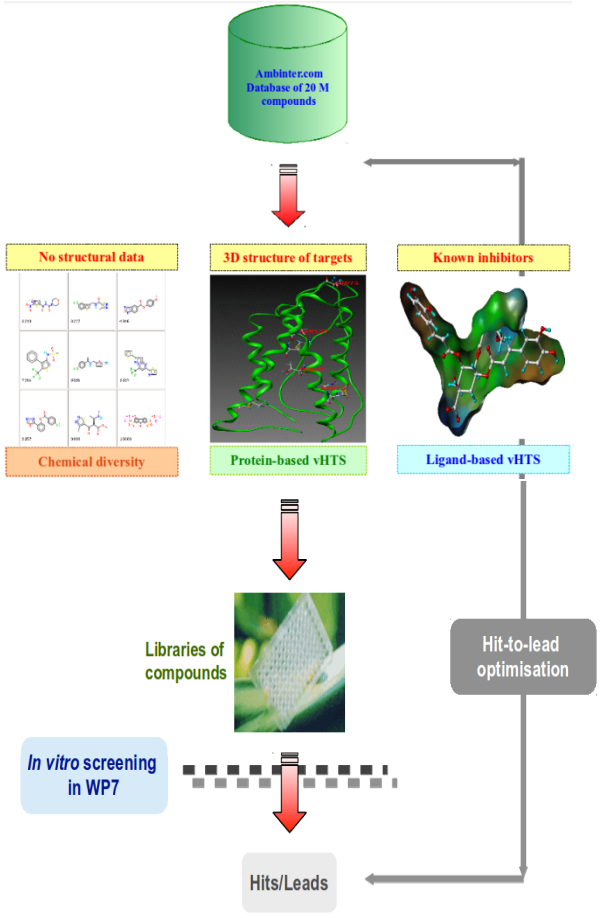
bioluminescence assay was developed for screening. Proteins of two novel targets were successfully purified. DSF screening assay were established for one. 5000 compounds of synthetic library and 100 natural compounds were screened on the novel targets. **41 actives** were **selected** and **15 hits identified**.

Finally, **21** compounds, selected by vHTS (WP8) for targeting two essential targets identified in WP2, were assayed. For **six** of these compounds that showed significant growth inhibition activities on PAO1, protocols of optimization are underway.

WP8 - Structural characterization of the targets and virtual HTS of compound libraries

This WP aimed at identifying lead inhibitors of selected targets by virtual HT screenings (vHTS) in a rational drug discovery approach. To obtain 3D target structures for vHTS, two strategies were adopted in parallel: (i) determination of protein 3D structure by crystallography and (ii) homology modeling. Unfortunately, the first strategy was unsuccessful for the targets that were considered. Therefore, homology 3D modeling was performed whenever possible. Furthermore, Greenpharma established a procedure to score the relevancy of **3D structures** of target proteins starting from their amino-acid sequence; the **druggability** and **patentability** of targets were also considered. This procedure was applied to 332 *P. aeruginosa* proteins.

Importantly, experimental inputs from Università Vita e Salute, University of Nottingham, University of Tuebingen and University of Milan were crucial to prioritise and balance the novel targets for vHTS between essential and virulence factors. A well-established target such as DNA gyrase (new inhibitory site), was also selected to possibly accelerate the discovery of leads.



Ten vHTS campaigns were undertaken by Greenpharma using 25,000 original synthetic compounds from Actar-KDevExploratory, 20 million products from Ambinter.com and 150,000 natural compound structures available in Greenpharma database.

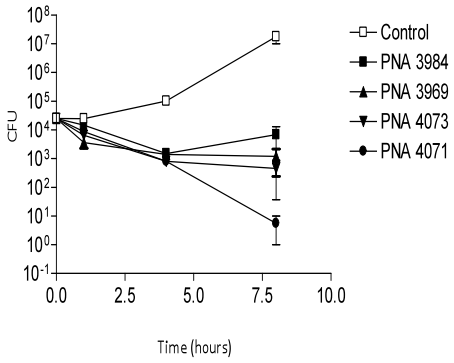
Several candidate inhibitors were identified by vHTS and assayed for activity in WP7. **Structure-Activity Relationship** (SAR) was explored for several compounds to prepare hit-to-lead optimisation. This consists in designing (computer-aided) similar derivatives from hits, yet with a certain degree of diversity between them, in order to generate a maximum of SAR data.

In parallel with 3D structure-based vHTS, chemoinformatic tools were used to design two libraries with top-level chemical diversity to maximise the probability to get hits in the assays performed in WP7. One **library** consisted of **natural compounds** from plants and micro-organisms (480 pure molecules); the other contained 7000 molecules from **synthetic or hemisynthetic compounds**. These libraries were used in *in vitro* screening without target structural bias (it is complementary to the protein-based approach) to explore thoroughly the possibility to inhibit select targets. This screening yielded **12 inhibitors** of **PqsA**, an important gene controlling QS and responsible for biofilm formation and virulence factors. Two most potent hits were retained for further chemical improvements. Evaluations of these new derivatives are on-going.

WP9 - Development of novel antisense antimicrobials

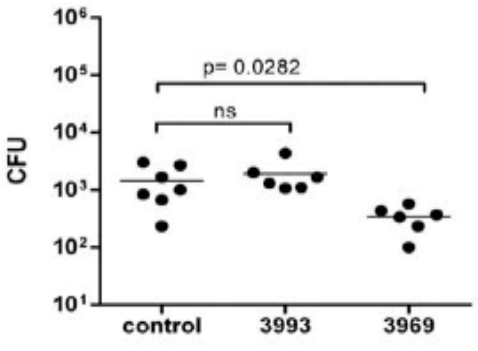
One alternative strategy of NABATIVI was to explore **antisense technology** for targeted inhibition of gene expression, and to exploit this new knowledge for discovery of **novel peptide nucleic acid** (PNA) based antibiotics against *P. aeruginosa.*

Studies of PNA oligomers and PNA-peptide conjugates in *E.coli* and *S.aureus* had very clearly shown that insufficient bacterial uptake is the major limiting factor for the efficacy of PNA antibacterials (Good, 2001). The use of carrier peptides such as the KFFKFFKFFK peptide can to some extent alleviate this problem in *E. coli* but is far from the optimal solution. However, it was not known which carrier peptides could be effective *P. aeruginosa.* Thus for discovery of novel antimicrobial agents against *P. aeruginosa* a variety of both gene and sequence targets as well as delivery agents was explored in the WP. The starting point iwas the essential genes *acpP* and *ftsZ* and the KFFKFFKFFK peptide carrier already validated in *E. coli,* as well as the biofilm associated *psq*A gene identified in key pathways of QS.



University of Copenhagen has designed and synthesized more than 70 PNA-peptide conjugates using a variety of carrier peptides and mRNA target sequences, and characterized these in terms of antibacterial activity against *P. aeruginosa* as well as in terms of mechanism of action. This screening and optimization resulted in identification of four **PNA-peptide conjugates** (PNA3969, PNA3984, PNA4071 & PNA4073) (targeting *acpP* and *ftsZ, respectively)* exhibiting potent antibacterial activity (MIC values of 0.6 – 2 µM) against three medically relevant *P. aeruginosa* strains (PA01, PA14 & LESB58), and all showed (at least partly) **bactericidal activity**.

On the basis of lower toxicity to eukaryotic cells in culture one of these (PNA3969) was selected for *in vivo* experiments in a lung infection mouse model, and data from this study demonstrate ***in vivo* antibacterial activity** of this PNA compared to untreated control or a similar sequence mismatch control PNA (PNA3993). A PNA-peptide conjugate (PNA4064) down regulating *pqs*A was also discovered and characterized, showing to down regulate quorum sensing signals and inhibit biofilm formation.



PNA efficacy in murine model of respiratory infection

In addition, PNA peptide conjugates targeting mRNA of genes selected by NABATIVI consortium (PNAs 4359 & 4360) have been synthesized and are being tested in *in vitro* and *in vivo* models (manuscript in preparation).

In conclusion this work package has identified an antisense antimicrobial lead compound showing potent antibacterial activity in vitro as well as in vivo activity against *P. aeruginosa.*

WP10 - Efficacy and safety of selected antimicrobials

The long-term goal of NABATIVI is to develop a new class of anti-bacterial drugs which are active against sensitive and multi-drug resistant *P. aeruginosa* and other gram-negative strains. Thus, the main objective of this WP was to test the activity of selected antimicrobials against a panel of multi-drug resistant *P. aeruginosa* strains. **POL7001**, a potent **antibiotic** acting specifically against *P. aeruginosa* with a **new mode of action** (see WP3) was chosen for this purpose. The efficacy of POL7001 was demonstrated ***in vitro*** by assessing its **antimicrobial activity** against a selected panel of sensitive and multi-drug resistant (MDR) *P. aeruginosa* clinical isolates from CF patients. In particular, the Minimun Inhibitory Concentration (**MIC**), the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation, was measured. MICs are important in clinical microbiology and laboratories to monitor the activity of new antimicrobial agents and to confirm resistance of microorganisms to an antimicrobial agent. The MICs of POL7001 and other antibiotics clinically used to treat *P. aeruginosa* infections, the so called reference antibiotics, were determined against the strains collection of the Hannover CF center (see WP5). MICs were determined for POL7001, ciprofloxacin, meropenem, ceftazidime, colistin, gentamicin, tobramycin, and imipenem. MICs for POL7001 were particularly low, ranging between < 0.0005 - 0.125 µg/mL for all isolates, indicating a potent *in vitro* activity of POL7001. For the same isolates, MICs of reference antibiotics were comparable or much higher (ranges 0.03->8 µg/mL). Over time, many of the *P. aeruginosa* isolates from the patients became resistant to two or more antibiotics while remaining sensitive to POL7001. There was no difference in activity of POL7001 against mucoid and non-mucoid or hypermutable isolates, which tend in general to become resistant to several antibiotic classes. This is in agreement to the new mode of action identified for POL7001.

***Pseudomonas aeruginosa* strains**	**POL7001**	**Colistin**	**Gentamicin**	**Ciprofloxacin**	**Ceftazidime**	**Meropenem**	**Tobramycin**	**Imipenem**
Reference strain for antibiotics (ATCC 27853)	0.03	0.25	0.5	0.25	2	2	0.25	4
Reference laboratory strain (PAO1)	**0.125**	**0.125**	**1**	**0.125**			**0.25**	
Clinical strain 1 (RP73)	**0.125**	**0.5**	**8**	**0.5**	**> 8**	**8**	**2**	**> 8**
Clinical strain 2 (AA43), mucoid	0.06	0.5	4	0.25	8	2	1	0.5
Clinical strain 3 (AA44)	0.03	0.125	4	0.5	1	1	1	0.5
Clinical strain 4 (SG2), hypermutable	0.06	0.125	> 8	0.06	8	0.25	4	0.25
Clinical strain 5 (BT1), hypermutable	0.125	0.5	> 8	0.06	2	0.5	> 8	0.5
Clinical strain 6 (TR66)	0.06	0.5	8	0.25	> 8	4	2	8
Clinical strain 7 (KK71)	0.03	0.5	4	1	8	> 8	0.5	> 8
Clinical strain 8 (KK72)	0.03	0.5	4	1	8	> 8	0.5	> 8
Clinical strain 9 (MF51)	0.06	0.125	> 8	1	8	> 8	4	> 8
Clinical strain 10 (MF52)	0.03	0.125	> 8	1	2	0.5	8	0.5

MIC < 0.125

0.125 ≤ MIC < 0.5

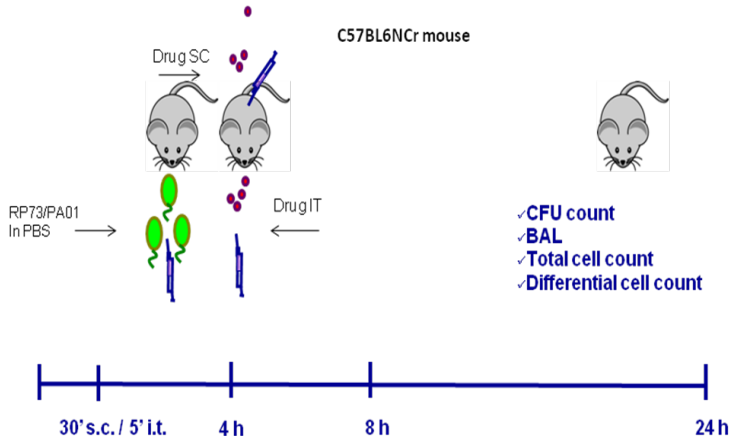
0.5 ≤ MIC < 8

MIC ≥ 8

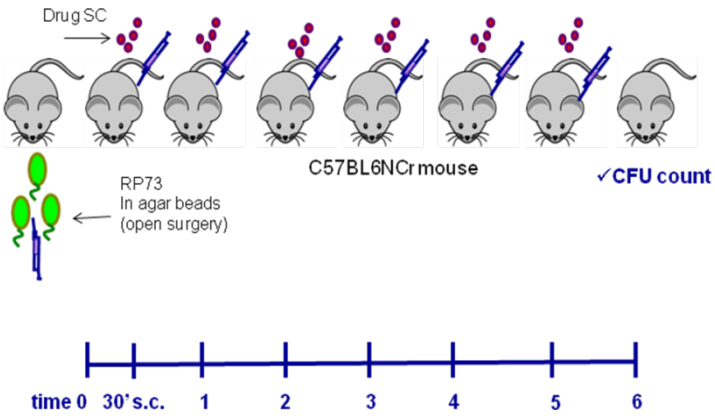
Next, Polyphor Ltd evaluated the **safety** of selected antimicrobials. For this purpose, the *in vitro* **ADMET properties** of POL7001 were measured. POL7001 was not cytotoxic against

COS 7 and Hela cell lines at concentrations of 100mg/ml. In addition, POL7001 was not hemolytic against red blood cells at 100mg/ml indicating no potential for toxicity against mammalian cells. The metabolism stability of POL7001 was measured in presence of liver microsomes from different species. Microsomes are valuable tools for investigating the metabolism of compounds (enzyme inhibition, clearance and metabolite identification) *in vitro*. POL7001 is metabolically stable towards liver microsomes, indicating that the compound will not be readily metabolised in the liver. The stability of POL7001 was also measured in presence of plasma from different species. POL7001 was stable towards enzymatic degradation in the plasma, indicating that the compound will remain in the blood stream after administration to exert its antimicrobial activity. In summary, the ADMET properties of POL7001 are favourable and support further development of this novel antibiotic.

To show ***in vivo* efficacy** of POL7001, the compound was tested in murine models of acute lung infection induced by *P. aeruginosa*. Mice were infected with bacteria by intra-tracheal surgery and treated either with POL7001 or ciprofloxacin as control antibiotic. Different routes of administration were tested, in order to reproduce standard clinical procedures. In particular, the antibiotics were administered either subcutaneously (s.c.) or intra-tracheally (i.t.), at different doses and at different time points post infection. In all studies POL7001 showed comparable or significantly better efficacy than ciprofloxacin, especially when administered intra-tracheally.



The *in vivo* efficacy of POL7001 was tested also in a chronic infection model with bacteria embedded in agar beads. These immobilising agents provide the microaerobic/anaerobic conditions that allow bacteria to grow in the form of microcolonies, similarly to the growth in the mucus of CF patients. After the infection, mice were treated once daily for six days by s.c. administration of POL7001 or ciprofloxacin. After 6 days of infection, mice treated s.c. with POL7001 displayed a slight but significant decrease in bacterial load Interestingly, ciprofloxacin did not show a significant efficacy in this model. Considering that bacteria in this microenvironment are particularly difficult to treat, the efficacy results obtained with POL7001 are encouraging.



POL7001 reduced *P. aeruginosa* bacterial counts in murine models of acute and chronic respiratory infection. The efficacy studies *in vivo* support the pulmonary administration as a possible **therapeutic approach** for the treatment of lung infections with POL7001.

Potential Impact

Improving human health and impact on the economic cost of antimicrobial resistance. *P. aeruginosa* infections are a social burden and have a strong impact on the budgets of the National Health Systems. Due to the resistance to antibiotic treatment, two-thirds of nosocomial infections would be preventable by the development of new antibacterial principles. A major achievement of NABATIVI project was the discovery of a novel class of antibiotics against Gram-negative *Pseudomonas* with a novel mode of action. Inspired by the natural antimicrobial peptide protegrin I, a hit-to-lead optimisation program followed by a multidimensional strategy to optimize ADMET properties and *in vivo* efficacy in murine septicemia infection models, led to the identification of **POL7080**, which was nominated as a **clinical candidate**. POL7080 and its analogues represent a new antibiotic class (PEMdrug antibiotic) with a novel mode of action and an excellent safety and efficacy profile. The target gene (LptD) was identified during the drug discovery process. Efficacy of POL7001, a close analogue of POL7080, in murine models of pneumonia was confirmed. On November 4, 2013 Polyphor Ltd and Roche announced that they have entered into an exclusive worldwide license agreement to develop and commercialize POL7080 for patients suffering from bacterial infections caused by *P. aeruginosa*. Antimicrobial resistance represents a major threat to public health worldwide, leading to 25,000 deaths and related costs of over €1.5 billion in healthcare expenses and productivity losses in the European Union alone each year. *P. aeruginosa* accounts for one in every 10 hospital-acquired infections in the US and is listed as one of the six most dangerous drug-resistant microbes. Over 15% of *P. aeruginosa* isolates were resistant to at least three classes of antibiotics and close to five percent were resistant to all five classes under surveillance. Development of a new antibiotic from this class will enable the prescription of an efficacious drug to patients suffering from serious lung infections by *P. aeruginosa* where other existing antibiotics exhibit no or insufficient activity due to multidrug resistance. The development and validation of new therapies fostered by NABATIVI results are expected to impact on health promotion and prevention as well as to contribute significantly to the sustainability of efficient **health care systems**. In addition, the improved therapeutic strategies promoted by the NABATIVI results are expected to impact on the budgets of the National Health Systems lowering significantly the actual **economic cost** of the antibiotic resistance. Every treatment that substantially lowers *P. aeruginosa* prevalence in CF, as well as exerts beneficial effects on the patient's clinical status, is cost-effective compared with conventional antibiotic therapy for chronically infected CF patients. The reduction in the number of infections caused by *P. aeruginosa* would lead to lower number of hospital admissions with great consequences not only for patient quality of life, but also in indirect costs, with a decrease in the number of lost work days.

Reinforcing the competitiveness and boosting the innovative capacity of European health-related SMEs and businesses. A general aim of FP7-HEALTH Theme 1 was "increasing the competitiveness and boosting the innovative capacity of European health-related industries and businesses". As reported in I.1 Policy Context – Work Programme FP7-HEALTH Theme 1, "in line with the European strategy on life sciences and biotechnology and the Lisbon objectives, this Theme help increase the competitiveness of

European health care biotechnology and medical technology sectors where research intensive SMEs are the main economic drivers and pharmaceutical industries". As mentioned above, we witness the departure of many large pharmaceutical companies from antibacterial drug discovery for several economic reasons. In the case of NABATIVI, innovative **SMEs** filled the gap left by the migration of big pharmaceuticals out of the antibacterial sector and were the main economic drivers in the field. NABATIVI outcomes impacted positively the **competitiveness, innovative capacity, and businesses** of the involved SMEs relative to the wide market of antibiotics and provided them with the chance to become **leaders** in the field of **antibiotic discovery**.

Integrated multidisciplinary research at European level and exploitation of post-genomic information: Another aim of FP7-HEALTH Theme 1 was "to stimulate and sustain multidisciplinary basic biomedical research where large scale collaboration at the EU level is essential to exploit the full potential of post-genomic information to underpin applications to human health" (I.1 Policy Context – Work Programme FP7-HEALTH Theme 1). The integrated steps which supported the strategy of NABATIVI spanned **different disciplines** such as molecular microbiology and genetics, genomics, chemistry, biochemistry, structural biology, cell biology, bioinformatics, clinical research, epidemiology, and high-technologies related to the high-throughput screenings of compound libraries. Furthermore, the identification of novel targets was based on genetic technologies which deeply rely on the post-genomic information. For this configuration, the NABATIVI project matched also the objectives of the topic HEALTH-2007-2.1.2-5 (Multidisciplinary fundamental genomics and molecular biology approaches to study basic biological processes relevant to health and diseases) for the study of basic biological processes in bacterial such essential and virulence functions, which was relevant to the discovery of new and urgently needed antibiotics.

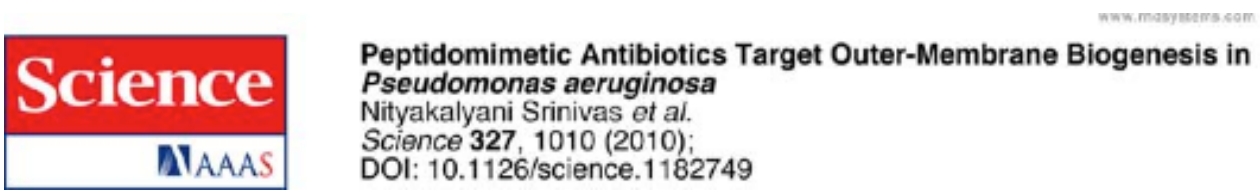
To support the high content of multidisciplinary research required for objective achievements NABATIVI project gathers academic and enterprise scientists from six European Countries bringing together multidisciplinary expertise. Therefore, the project promoted the **integration** of **European Excellence** in the field of antimicrobial drug resistance. The cooperative aspect in this proposal has undoubtedly facilitated such efforts devoted to the fight against the antibacterial drug resistance. Furthermore, the consolidation of NABATIVI partnership during the development of the project will lead to other collaborative research programmes between the partners to further progress the science in the field basing on NABATIVI outcomes, such as, for instance, projects focused on i) improvement and optimization of the lead compounds to increase their efficacy, ii) improved genetic technologies for genome-wide detection of virulence and essential genes, iii) genetic tools for defining and tracking the mode of action on novel antibacterial agents iv) process improvement of high-throughput screenings of compound libraries and antibacterial drug design.

Dissemination Activities & Exploitation of Results

Publications and meetings. NABATIVI has been widely disseminated both to the scientific research community and the general public. **23 publications** in refereed journals of international prestige have been achieved. NABATIVI has been promoted at **80 scientific meetings** by Partners of the consortium. Since the discovery of POL7080 as novel antibiotic with a potential direct impact on public health, **press release** has been organized by SMEs (http://www.polyphor.com/media/press-releases). In particular we point the attention on tthee press releases:

- February 19, 2010 - Polyphor discovers a new class of antibiotics with a novel mode of action. New weapon in the fight against multi-drug resistant bacteria published in "Science".
- March 4, 2013 - Polyphor reports successful Phase I results for its *Pseudomonas* selective antibiotic POL7080
- November 4, 2013- Polyphor and Roche join efforts to combat multidrug-resistant bacterial infections

Under the terms of the agreement, Roche will make an upfront payment of CHF 35 million to Polyphor as well as payments upon reaching certain development, regulatory and commercial milestones, potentially up to CHF 465 million. In addition, Polyphor is entitled to receive tiered double-digit royalties on product sales. Polyphor will retain the option to co-promote an inhaled formulation of POL7080 in Europe. The transaction is subject to customary regulatory clearances including termination of the applicable Hart-Scott-Rodino waiting period.



Furthermore, NABATIVI partners were involved in meeting with other EU projects. A **joint meeting** with AEROPATH consortium was organized in Bruxelles on March 2011 while a European Inter-network meeting was organized with Coordinators of AEROPATH, AntiPAthoGN, Divinocell, EUR-INTAFAR, PAR, Pneumopath, and UK Bacwan in Harzè on October 2010. These meetings gave the opportunity to disseminate NABATIVI results and set the basis for collaborative studies.

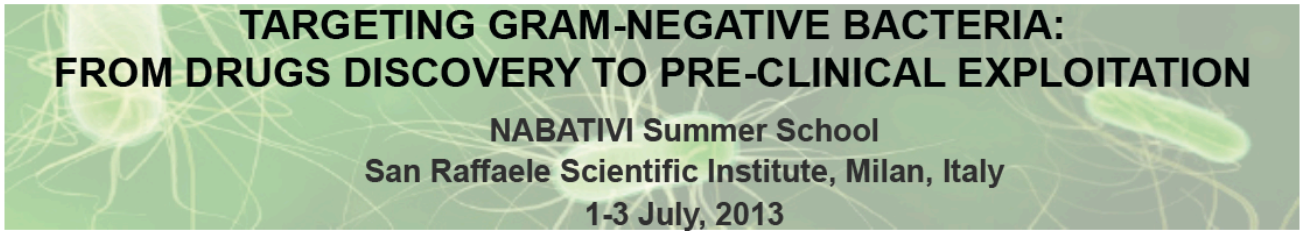
The web server of the Consortium, has been developed (**www.nabativi.org**) and played a central role for communication, training, and for the dissemination and exploitation of results.

Education and training of young researchers has been an important part of dissemination of the knowledge generated by NABATIVI. **Young postdoctoral** and **PhD students** played a fundamental role in the progress of the NABATIVI project and in the dissemination of novel results in the field of antibacterial drug discovery throughout Europe. The Consortium provided the young researchers with education and training on antibacterial drug discovery, with a focus on **multidisciplinarity**. PhD students and postdoctoral fellows engaged by the network participated in master-classes and international schools and presented their work at targeted international conferences. Such overall multidisciplinary education provided the young investigators with a global scientific view that enhanced their personal capabilities as potential future entrepreneurs.

The NABATIVI **summer school** named “Targeting Gram-Negative Bacteria: from Drug Discovery to Pre-clinical Exploitation” was organised at San Raffaele Scientific Institute (Milano, Italy) on July 1-3, 2013. This summer school brought together researchers from academia, industry and clinicians to discuss novel strategies for bacterial targets identification in the area of antibiotic discovery, with a focus on results from EU funded projects. A combination of lectures and practical sessions provided participants with background knowledge and a chance to get hands-on with tools employed. In particular, the lectures focused on:

- advanced genomic approaches for identification of virulence targets in gram-negative bacteria
- modelling infection in different model systems including human cell lines, non-mammalian and mouse models
- identification of novel drugs from the screening of chemical libraries against specifically selected targets and the characterisation of the mode of action of novel drugs with antibacterial activity

A selection of 20 **young scientists** from different EU countries who were working or planning to be involved in the field of anti-bacterial research participated to the summer school



4.2 Use and dissemination of foreground

Section A (public)

- Template A1: List of all scientific (peer reviewed) publications relating to the foreground of the project.

NO.	Title	Main author	Title of the periodical or the series	Number, date or frequency	Publisher	Year of publication	Relevant pages	Permanent identifiers[2] (if available)	Is/Will open access[3] provided to this publication?
1	Cystic Fibrosis-niche adaptation of Pseudomonas aeruginosa reduces virulence in multiple infection hosts	Lorè NI, Cigana C, De Fino I, Riva C, Juhas M, Schwager S, Eberl L, Bragonzi A	PLoS One	7	Public Library of Science	25/04/2012	35648	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0035648	Yes
2	Inhibition of lipopolysaccharide transport to the outer membrane in Pseudomonas aeruginosa by peptidomimetic antibiotics	Werneburg M, Zerbe K, Juhas M, Bigler L, Stalder U, Kaech A, Ziegler U, Obrecht D, Eberl L, Robinson JA	Chembiochem : a European journal of chemical biology	13	Wiley-VCH Verlag	13/08/2012	1767	http://www.ncbi.nlm.nih.gov/pubmed/22807320	No
3	Essence of life: essential genes of minimal genomes	Juhas M, Eberl L, Glass JI	Trends in Cell Biology	21	Elsevier Limited	01/09/2011	562	http://www.ncbi.nlm.nih.gov/pubmed/21889892	No
4	Comparative profiling of Pseudomonas aeruginosa strains reveals differential expression of novel unique and conserved small	Ferrara S, Brugnoli M, De Bonis A, Righetti F, Delvillani F, Dehò G, Horner D, Briani F, Bertoni G	PLoS One	7	Public Library of Science	10/05/2012	36553	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0036553	Yes

[2] A permanent identifier should be a persistent link to the published version full text if open access or abstract if article is pay per view) or to the final manuscript accepted for publication (link to article in repository).
[3] Open Access is defined as free of charge access for anyone via Internet. Please answer "yes" if the open access to the publication is already established and also if the embargo period for open access is not yet over but you intend to establish open access afterwards.

	RNAs								
5	Analysis of Pseudomonas aeruginosa cell envelope proteome by capture of surface-exposed proteins on activated magnetic nanoparticles	Vecchietti D, Di Silvestre D, Miriani M, Bonomi F, Marengo M, Bragonzi A, Cova L, Franceschi E, Mauri P, Bertoni G	PLoS One	7	Public Library of Science	30/11/2012	51062	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0051062	Yes
6	Murine models of acute and chronic lung infection with cystic fibrosis pathogens	Bragonzi A	International Journal of Medical Microbiology	8	Urban und Fischer Verlag GmbH und Co. KG	01/12/2010	584	http://www.ncbi.nlm.nih.gov/pubmed/20951086	No
7	Quinolones: from antibiotics to autoinducers	Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Cámara M	FEMS Microbiology Reviews	35	Blackwell Publishing	01/03/2011	247	http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3053476/	Yes
8	Fatty acid-mediated signalling between two Pseudomonas species	Fernández-Piñar R, Espinosa-Urgel M, Dubern JF, Heeb S, Ramos JL, Cámara M	Environmental Microbiology Reports	4	Wiley-Blackwell	01/08/2012	417	http://onlinelibrary.wiley.com/doi/10.1111/j.1758-2229.2012.00349.x/abstract	No
9	Peptidomimetic antibiotics target outer-membrane biogenesis in Pseudomonas aeruginosa	Srinivas N, Jetter P, Ueberbacher BJ, Werneburg M, Zerbe K, Steinmann J, Van der Meijden B, Bernardini F, Lederer A, Dias RL, Misson PE, Henze H, Zumbrunn J, Gombert FO, Obrecht D, Hunziker P, Schauer S, Ziegler U, Käch A, Eberl L, Riedel K, DeMarco SJ, Robinson JA	Science	Vol. 327. no. 5968	American Association for the Advancement of Science	19/02/2010	1010-1013	http://www.sciencemag.org/content/327/5968/1010.long	Yes
10	Fighting Back: Peptidomimetics as a New Weapon in the Battle Against Antibiotic Resistance	Bragonzi A	Science Translational Medicine	Volume 2 Issue 21	American Association for the Advancement of Science	03/03/2010	21	http://stm.sciencemag.org/content/2/21/21ps9.abstract	Yes
11	Pseudomonas aeruginosa Exploits Lipid A and Muropeptides Modification as a Strategy to Lower Innate	Cigana C, Curcurù L, Leone MR, Ieranò T, Lorè NI, Bianconi I, Silipo A, Cozzolino F, Lanzetta R, Molinaro A, Bernardini ML,	PLoS ONE	4(12)	PLoS	23/12/2009	8439	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0008439	Yes

		Bragonzi A							
12	Emerging New Therapeutics Against Key Gram-Negative Pathogens	Obrecht D, Bernardini F, Dale G, Dembowsky K	Ann. Rep. Med. Chem.	46		15/09/2011	245-262		No
13	High confidence prediction of essential genes in Burkholderia cenocepacia	Juhas M, Stark M, von Mering C, Lumjiaktase P, Crook DW, Valvano MA, Eberl L	PLoS One	7	Public Library of Science	29/06/2012	40064	http://www.plosone.org/article/info:doi/10.1371/journal.pone.0040064	Yes
14	The Pseudomonas aeruginosa quinolone quorum sensing signal alters the multicellular behaviour of Pseudomonas putida KT2440	Fernández-Piñar R, Cámara M, Dubern JF, Ramos JL, Espinosa-Urgel M	Res. Microbiol.	162 (8)		20/08/2011	773-81	http://www.ncbi.nlm.nih.gov/pubmed/21742029	Yes
15	Antibiotic pressure compensates the biological cost associated to Pseudomonas aeruginosa hypermutable phenotypes in vitro and in a murine model of chronic airway infection	Alcalá-Franco B, Montanari S, Cigana C, Bertoni G, Oliver A, Bragonzi A	JAC	67(4)		09/11/2011	962-9	http://jac.oxfordjournals.org/content/67/4/962.long	Yes
16	Positive signature-tagged mutagenesis in Pseudomonas aeruginosa: tracking patho-adaptive mutations promoting airways chronic infection	Bianconi I, Milani A, Cigana C, Paroni M, Levesque RC, Bertoni G, Bragonzi A	PLoS Pathog	7(2)		14/02/2011	1001270	http://www.plospathogens.org/article/info%3Adoi%2F10.1371%2Fjournal.ppat.1001270	Yes
17	Dampening host sensing and avoiding recognition in Pseudomonas aeruginosa pneumonia	Cigana C, Lorè NI, Bernardini ML, Bragonzi A	J Biomed Biotechnol.	2011		05/07/2011	852513	http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3139209/	Yes
18	Essential genes as antimicrobial targets and cornerstones of synthetic biology	Juhas M, Eberl L, Church GM	Trends in Biotechnology	30	Elsevier Limited	01/11/2012	601	http://www.ncbi.nlm.nih.gov/pubmed/22951051	No
19	Potent Antibacterial Antisense Peptide-Peptide Nucleic Acid Conjugates Against Pseudomonas Aeruginosa	Ghosal A, Nielsen PE	Oligonucleotides	22	Mary Ann Liebert Inc.	02/10/2012	323	http://www.ncbi.nlm.nih.gov/pubmed/23030590	No

20	Role of SbmA in the Uptake of Peptide Nucleic Acid (PNA)-Peptide Conjugates in E. Coli	Ghosal A, Vitali A, Stach JE, Nielsen PE	ACS Chemical Biology	8	American Chemical Society	15/02/2013	360	http://www.ncbi.nlm.nih.gov/pubmed/23138594	No
21	TgpA, a protein with a eukaryotic-like transglutaminase domain, plays a critical role in the viability of Pseudomonas aeruginosa	Milani A, Vecchietti D, Rusmini R, Bertoni G	PLoS One	7	Public Library of Science	27/11/2012	50323	http://www.plosone.org/article/info:doi/10.1371/journal.pone.0050323	Yes
22	Analysis of Pseudomonas aeruginosa cell envelope proteome by capture of surface-exposed proteins on activated magnetic nanoparticles	Vecchietti D, Di Silvestre D, Miriani M, Bonomi F, Marengo M, Bragonzi A, Cova L, Franceschi E, Mauri P, Bertoni G	PLoS One	7	Public Library of Science	30/11/2012	51062	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0051062	Yes
23	Comparative profiling of Pseudomonas aeruginosa strains reveals differential expression of novel unique and conserved small RNAs	Ferrara S, Brugnoli M, De Bonis A, Righetti F, Delvillani F, Dehò G, Horner D, Briani F, Bertoni G	PLoS One	7	Public Library of Science	10/05/2012	36553	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0036553	Yes
24	Novel targets for antimicrobial molecules in Pseudomonas aeruginosa: identification and characterization of an essential membrane eukaryotic-like transglutaminase	Vecchietti D, Milani A, Rusmini R, and Bertoni G	Pediatric Pulmonology	47	Wiley-Liss Inc.	01/09/2012	340		No
25	Analysis of cell envelope proteome of Pseudomonas aeruginosa by capture of surface-exposed proteins on activated magnetic nanoparticles	Vecchietti D, Miriani M, Di Silvestre D, Bonomi F, Marengo M, Mauri P, and Bertoni G	Pediatric Pulmonology	47	Wiley-Liss Inc.	01/09/2012	341		No
26	Tuned magnetic nanoparticles for studying surface-exposed proteins in bacterial cells.	Bonomi F, Miriani M, Marengo M, Vecchietti D, Cova L,. Bragonzi A, Di Silvestre D, Mauri P, Franceschi E and G. Bertoni	FEBS Journal	279	Blackwell Publishing	01/09/2012	451		No

27	Structural studies of β-hairpin peptidomimetic antibiotics that target LptD in Pseudomonas sp.	Schmidt J, Patora-Komisarska K, Moehle K, Obrecht D, Robinson JA	Bioorganic and Medicinal Chemistry	21	Elsevier Limited	15/09/2013	5806	http://www.ncbi.nlm.nih.gov/pubmed/23932450	No
28	Cystic fibrosis-adaptation of Pseudomonas aeruginosa modulates innate immune system detection and tissue damage control	Cigana C, Lorè NI, Riva C, De Fino I, Bragonzi A	Pediatric Pulmonology	47	Wiley-Liss Inc.	01/09/2012	336		No
29	Evaluation of host immune system evasion and induction of damage during Pseudomonas aeruginosa adaptation to cystic fibrosis lung	Cigana C, Lorè NI, Eberl L, Bragonzi A	Pediatric Pulmonology	33	Wiley-Liss Inc.	01/09/2010	346		No
30	Adaptation of P. aeruginosa in cystic fibrosis airways affects the host immune-response	Cigana C, Lorè NI, De Fino I, Schwager S, Juhas M, Eberl L, Bragonzi A	Pediatric Pulmonology	34	Wiley-Liss Inc.	01/09/2011	320		No
31	A shotgun antisense approach to the identification of novel essential genes in *Pseudomonas aeruginosa*.	Rusmini R., Vecchietti D., Macchi R., Vidal-Aroca F. and Bertoni G.	BMC Microbiology						
32	Two approaches of functional genomics in the opportunistic pathogen *Pseudomonas aeruginosa*	Milani A.	PhD thesis	April 2010	Unknown	2010		Unknown	no

TEMPLATE A2: LIST OF DISSEMINATION ACTIVITIES								
NO.	Type of activities[4]	Main leader	Title	Date/Period	Place	Type of audience[5]	Size of audience	Countries addressed
1	Presentations	UNIVERSITAET ZUERICH	Gordon Conference on Bioorganic Chemistry	14/06/2009	Andover, USA	Scientific community (higher education, Research)	200	USA, Europe
2	Oral presentation to a scientific event	Københavns Universitet	11th Int. Conf. amino acids, peptides proteins	03/08/2009	Wien, Austria	Scientific community (higher education, Research)	150	Europe
3	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	12th International Conference on Pseudomonas	15/08/2009	Hannover, Germany	Scientific community (higher education, Research)	400	Europe, USA
4	Web sites/Applications	UNIVERSITA VITA-SALUTE SAN RAFFAELE	www.nabativi.org	01/09/2009	Milan, Italy	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias		Europe, USA
5	Presentations	UNIVERSITA DEGLI STUDI DI MILANO	23rd North-American Cystic Fibrosis Conference	16/10/2009	Minneapolis, USA	Scientific community (higher education, Research)	1000	USA, Europe
6	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	23rd North-American Cystic Fibrosis Conference	16/10/2009	Minneapolis, USA	Scientific community (higher education, Research)	1000	USA, Europe
7	Oral presentation to a	UNIVERSITAET ZUERICH	Symposium and European workshop on Foldamers	25/01/2010	Bordeaux, France	Scientific community (higher	150	Europe

[4] A drop down list allows choosing the dissemination activity: publications, conferences, workshops, web, press releases, flyers, articles published in the popular press, videos, media briefings, presentations, exhibitions, thesis, interviews, films, TV clips, posters, Other.

[5] A drop down list allows choosing the type of public: Scientific Community (higher education, Research), Industry, Civil Society, Policy makers, Medias, Other ('multiple choices' is possible).

	scientific event					education, Research)		
8	Posters	UNIVERSITA DEGLI STUDI DI MILANO	Pseudomonas2010	28/01/2010	Milan, Italy	Scientific community (higher education, Research)	400	Europe
9	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	Pseudomonas2010	28/01/2010	Milan, Italy	Scientific community (higher education, Research)	400	Europe
10	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Gordon Research Conference on Chemistry and Biology of Peptides	01/03/2010	Ventura, USA	Scientific community (higher education, Research)	150	USA, Europe
11	Presentations	UNIVERSITAET ZUERICH	Gordon Conference on Peptides	10/03/2010	Ventura, USA	Scientific community (higher education, Research)	200	USA, Europe
12	Articles published in the popular press	UNIVERSITA VITA-SALUTE SAN RAFFAELE	http://www.nabativi.org/index.php?pos=950&nid=42&tid=	13/03/2010	Verona, Italy	Scientific community (higher education, Research) - Civil society		Europe
13	Thesis	UNIVERSITA DEGLI STUDI DI MILANO	PdD thesis dissertation at the University of Milan, Italy	22/04/2010	Milan, Italy	Scientific community (higher education, Research)	50	Europe
14	Oral presentation to a scientific event	Københavns Universitet	Medichem-2010	17/05/2010	Bejing, China	Scientific community (higher education, Research)	50	USA, Asia, Europe
15	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the University of Freiburg;	17/05/2010	Freiburg, Germany	Scientific community (higher education, Research)	50	Europe
16	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	ASM 110th General Meeting	23/05/2010	San Diego, USA	Scientific community (higher education, Research)	3000	USA, Europe
17	Posters	UNIVERSITA DEGLI STUDI DI MILANO	ASM 110th General Meeting	23/05/2010	San Diego, USA	Scientific community (higher education, Research)	3000	USA, Europe
18	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the Free University of Berlin and Leibniz-Institut für Molekulare Pharmakologie	31/05/2010	Berlin, Germany	Scientific community (higher education, Research)	50	Europe
19	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Naples Workshop on Bioactive Peptides	05/06/2010	Naples, Italy	Scientific community (higher education, Research)	150	Europe
20	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the University of Tuebingen	22/07/2010	Tuebingen, Germany	Scientific community (higher education, Research)	50	Europe

21	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the University of Frankfurt; Frankfurt	22/09/2010	Frankfurt, Germany	Scientific community (higher education, Research)	50	Europe
22	Presentations	UNIVERSITAET ZUERICH	OGMBT Annual Meeting	29/09/2010	Wienna, Austria	Scientific community (higher education, Research)	100	Europe
23	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	24th North-American Cystic Fibrosis Conference	21/10/2010	Baltimore, USA	Scientific community (higher education, Research)	1000	USA, Europe
24	Oral presentation to a scientific event	Københavns Universitet	EuroPEPTIDES	16/11/2010	Barcelona, Spain	Scientific community (higher education, Research)	100	Europe
25	Presentations	UNIVERSITA DEGLI STUDI DI MILANO	8th Convention of the Italian Cystic Fibrosis Foundation	03/12/2010	Verona, Italy	Scientific community (higher education, Research)	50	Europe
26	Presentations	UNIVERSITAET ZUERICH	Biolloquium der TU Munchen	14/12/2010	Freising, Germany	Scientific community (higher education, Research)	50	Europe
27	Presentations	UNIVERSITAET ZUERICH	Seminar at the University of Ghent	01/03/2011	Ghent, Belgium	Scientific community (higher education, Research)	50	Europe
28	Presentations	POLYPHOR AG	GDCh	21/03/2011	Saarbrücken, Germany	Scientific community (higher education, Research)	50	Europe
29	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the ETH-Zurich, Institute for Pharmaceutical Sciences	30/03/2011	Zurich, Switzerland	Scientific community (higher education, Research)	100	Europe
30	Presentations	UNIVERSITA VITA-SALUTE SAN RAFFAELE	ECFS Conference 2011	01/04/2011	Tirrenia, Italy	Scientific community (higher education, Research)	800	Europe, USA
31	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	EUCHEM Conference on Stereochemistry	03/05/2011	Brunnen, Switzerland	Scientific community (higher education, Research)	150	Europe
32	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	TOLL 2011	04/05/2011	Riva del Garda, Italy	Scientific community (higher education, Research)	400	Europe, USA
33	Posters	POLYPHOR AG	ECCMID Conference	07/05/2011	Milan, Italy	Scientific community (higher education, Research)	300	Europe
34	Presentations	Københavns Universitet	Peptide Vector Delivery Conference	19/05/2011	Tallinn, Estonia	Scientific community (higher education, Research)	75	Europe

35	Presentations	POLYPHOR AG	Venoms to Drugs Conference	19/05/2011	Heron Island, Australia	Scientific community (higher education, Research)	100	USA, Europe
36	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the University of Bern	27/05/2011	Bern, Germany	Scientific community (higher education, Research)	50	Europe
37	Presentations	Københavns Universitet	17th Conversation	14/06/2011	Albany, USA	Scientific community (higher education, Research)	200	USA
38	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the Max Planck Institut für Medizinische Forschung	16/06/2011	Heidelberg, Germany	Scientific community (higher education, Research)	50	Europe
39	Presentations	UNIVERSITAET ZUERICH	FEMS Conference	30/06/2011	Geneva, Switzerland	Scientific community (higher education, Research)	200	Europe
40	Posters	THE UNIVERSITY OF NOTTINGHAM	13th International Conference on Pseudomonas	04/09/2011	Sydney, Australia	Scientific community (higher education, Research)	400	USA, Europe
41	Posters	Københavns Universitet	13th International Conference on Pseudomonas	04/09/2011	Sydney, Australia	Scientific community (higher education, Research)	400	Europe, USA
42	Posters	UNIVERSITA DEGLI STUDI DI MILANO	SIMGBM 29th National Meeting	21/09/2011	Pisa, Italy	Scientific community (higher education, Research)	100	Europe
43	Presentations	UNIVERSITAET ZUERICH	Seminar at Institute of Molecular and Cell Biology, A*STAR Singapore	26/09/2011	Singapore, Singapore	Scientific community (higher education, Research)	100	USA, Europe
44	Presentations	Københavns Universitet	14th Int. Symp. Molecular Medicine	06/10/2011	Rhodes, Greece	Scientific community (higher education, Research)	100	Europe
45	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the University of Essen-Duisberg	26/10/2011	Essen-Duisberg, Germany	Scientific community (higher education, Research)	50	Europe
46	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the Max Planck Institut für Kohlenforschung	27/10/2011	Mühlheim an der Ruhr, Germany	Scientific community (higher education, Research)	50	Europe
47	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the University of Geneva	03/11/2011	Geneva, Switzerland	Scientific community (higher education, Research)	50	Europe
48	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	25th North-American Cystic Fibrosis Conference	03/11/2011	Anaheim, USA	Scientific community (higher education, Research)	1000	USA, Europe

49	Presentations	THE UNIVERSITY OF NOTTINGHAM	Annual Cystic Fibrosis Microbiology Consortium meeting	29/11/2011	Liverpool, UK	Scientific community (higher education, Research)	100	Europe
50	Posters	POLYPHOR AG	9th Convention of the Italian Cystic Fibrosis Foundation	01/12/2011	Verona, Italy	Scientific community (higher education, Research)	50	Europe
51	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	9th Convention of the Italian Cystic Fibrosis Foundation	01/12/2011	Verona, Italy	Scientific community (higher education, Research)	50	Europe
52	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Zing Natural Product Symposium	10/02/2012	Tenerife, Spain	Scientific community (higher education, Research)	150	Europe
53	Oral presentation to a scientific event	THE UNIVERSITY OF NOTTINGHAM	Biotechnologies (3CIBM)	15/03/2012	Tangier, Morocco	Scientific community (higher education, Research)	300	USA, Europe
54	Oral presentation to a scientific event	POLYPHOR AG	Gordon Research Conference	07/04/2012	Barga, Italy	Scientific community (higher education, Research)	100	Europe
55	Oral presentation to a scientific event	UNIVERSITA VITA-SALUTE SAN RAFFAELE	European Cystic Fibrosis Young Investigators Meeting	24/04/2012	Paris, France	Scientific community (higher education, Research)	100	Europe
56	Posters	UNIVERSITA DEGLI STUDI DI MILANO	2nd MoI Micro Meeting	25/04/2012	Wurzburg, Germany	Scientific community (higher education, Research)	300	Europe
57	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Centre of Applied Science for Health, ITT Dublin and the BioAT programme	10/05/2012	Dublin, Ireland	Scientific community (higher education, Research)	100	Europe
58	Oral presentation to a scientific event	POLYPHOR AG	Trends in Drug Research; 30th Noordwijkerhout-Camerino-Cyprus Symposium	13/05/2012	Amsterdam, Netherlands	Scientific community (higher education, Research)	75	Europe
59	Oral presentation to a scientific event	POLYPHOR AG	TIDES	23/05/2012	Las Vegas, USA	Scientific community (higher education, Research)	100	USA
60	Oral presentation to a scientific event	POLYPHOR AG	New Antimicrobials Workshop “New Compounds & New Strategies for Antimicrobials”	25/05/2012	Trieste, Italy	Scientific community (higher education, Research)	75	Europe
61	Oral presentation to a scientific event	UNIVERSITA VITA-SALUTE SAN RAFFAELE	35th European Cystic Fibrosis Conference	06/06/2012	Dublin, Ireland	Scientific community (higher education, Research)	600	Europe, USA
62	Posters	POLYPHOR AG	35th European Cystic Fibrosis Conference	06/06/2012	Dublin, Ireland	Scientific community (higher education, Research)	600	Europe, USA

63	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	35th European Cystic Fibrosis Conference	07/06/2012	Dublin, Ireland	Scientific community (higher education, Research)	600	Europe, USA
64	Oral presentation to a scientific event	THE UNIVERSITY OF NOTTINGHAM	35th European Cystic Fibrosis Conference	07/06/2012	Dublin, Ireland	Scientific community (higher education, Research)	600	Europe, USA
65	Oral presentation to a scientific event	Københavns Universitet	3rd Int. Symp.: Antimicrobial Peptides	13/06/2012	Lille, France	Scientific community (higher education, Research)	150	Europe, USA
66	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Invited seminar at the University of Erlangen	21/06/2012	Erlangen, Germany	Scientific community (higher education, Research)	50	Europe
67	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Seminar at the University of Greifswald	05/07/2012	Greifswald, Germany	Scientific community (higher education, Research)	50	Europe
68	Oral presentation to a scientific event	UNIVERSITA DEGLI STUDI DI MILANO	FEBS RNA Satellite Meeting	01/09/2012	Tavira, Portugal	Scientific community (higher education, Research)	300	Europe
69	Posters	UNIVERSITA DEGLI STUDI DI MILANO	22nd IUBMB/37th FEBS Congress	04/09/2012	Sevilla, Spain	Scientific community (higher education, Research)	300	Europe
70	Oral presentation to a scientific event	THE UNIVERSITY OF NOTTINGHAM	34th ESPEN Congress	08/09/2012	Barcelona, Spain	Scientific community (higher education, Research)	500	Europe
71	Oral presentation to a scientific event	UNIVERSITA VITA-SALUTE SAN RAFFAELE	Cytokines 2012, 10th Joint Annual Meeting	11/09/2012	Geneve, Switzerland	Scientific community (higher education, Research)	200	Europe
72	Oral presentation to a scientific event	THE UNIVERSITY OF NOTTINGHAM	Central European Symposium on Antimicrobials and Antimicrobial Resistant (CESAR)	23/09/2012	Primosten, Croatia	Scientific community (higher education, Research)	200	Europe
73	Oral presentation to a scientific event	UNIVERSITA DEGLI STUDI DI MILANO	12th Congress FISV, La Sapienza University of Rome	24/09/2012	Rome, Italy	Scientific community (higher education, Research)	300	Europe
74	Posters	UNIVERSITA DEGLI STUDI DI MILANO	12th Congress FISV, La Sapienza University of Rome	24/09/2012	Rome, Italy	Scientific community (higher education, Research)	300	Europe
75	Oral presentation to a scientific event	UNIVERSITAET ZUERICH	Max Bergmann Conference	01/10/2012	Velen, Germany	Scientific community (higher education, Research)	100	Europe
76	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	26th North-American Cystic Fibrosis Conference	11/10/2012	Orlando, USA	Scientific community (higher education, Research)	1000	USA, Europe

77	Posters	UNIVERSITA DEGLI STUDI DI MILANO	26th North-American Cystic Fibrosis Conference	11/10/2012	Orlando, USA	Scientific community (higher education, Research)	1000	USA, Europe
78	Oral presentation to a scientific event	POLYPHOR AG	Lecture at the 10th course on medicinal chemistry	18/10/2012	Leysin, Switzerland	Scientific community (higher education, Research)	50	Europe
79	Oral presentation to a scientific event	Københavns Universitet	EURO-Tides	27/11/2012	Berlin, Germany	Scientific community (higher education, Research)	100	Europe
80	Posters	THE UNIVERSITY OF NOTTINGHAM	Annual Cystic Fibrosis Microbiology Consortium Meeting	28/11/2012	Liverpool, UK	Scientific community (higher education, Research)	100	Europe
81	Oral presentation to a scientific event	THE UNIVERSITY OF NOTTINGHAM	Annual Cystic Fibrosis Microbiology Consortium Meeting	28/11/2012	Liverpool, UK	Scientific community (higher education, Research)	100	Europe
82	Oral presentation to a scientific event	UNIVERSITA VITA-SALUTE SAN RAFFAELE	10th Convention of the Italian Cystic Fibrosis Foundation	29/11/2012	Verona, Italy	Scientific community (higher education, Research)	75	Europe
83	Oral presentation to a scientific event	POLYPHOR AG	Lecture at University of Zurich	11/12/2012	Zurich, Switzerland	Scientific community (higher education, Research)	50	Europe
84	Oral presentation to a scientific event	UNIVERSITA VITA-SALUTE SAN RAFFAELE	European Cystic Fibrosis Young Investigators Meeting	27/02/2013	Paris, France	Scientific community (higher education, Research)	100	Europe
85	Oral presentation to a scientific event	POLYPHOR AG	Lecture at the Pharmazentrum, University of Basel	06/03/2013	Basel, Switzerland	Scientific community (higher education, Research)	50	Europe
86	Press releases	UNIVERSITA VITA-SALUTE SAN RAFFAELE	http://cordis.europa.eu/fetch?ACTION=D&SESSION=&DOC=1&TBL=EN_OFFR&RCN=6432&CALLER=OFFR_TM_EN	15/03/2013	Bruxelles, Belgium	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias		Europe
87	Oral presentation to a scientific event	POLYPHOR AG	SCT workshop: Biological relevant molecular diversity	04/04/2013	Paris, France	Scientific community (higher education, Research)	100	Europe
88	Press releases	POLYPHOR AG	http://www.nabativi.org/index.php?pos=950&nid=70&tid=	04/04/2013	Allschwil, Switzerland	Scientific community (higher education, Research) - Industry - Civil society - Medias		Europe, USA
89	Oral presentation to a scientific event	POLYPHOR AG	Drug Discovery Chemistry Meeting	17/04/2013	San Diego, USA	Scientific community (higher education, Research)	100	USA

90	Oral presentation to a scientific event	POLYPHOR AG	TIDES meeting	15/05/2013	Boston, USA	Scientific community (higher education, Research)	150	USA, Europe
91	Posters	UNIVERSITA DEGLI STUDI DI MILANO	3rd International Conference on Regulating with RNA in Bacteria	04/06/2013	Wurzburg, Germany	Scientific community (higher education, Research)	300	Europe
92	Oral presentation to a scientific event	UNIVERSITA VITA-SALUTE SAN RAFFAELE	World Research and Innovation Congress	06/06/2013	Brussels, Belgium	Scientific community (higher education, Research)	200	Europe
93	Oral presentation to a scientific event	THE UNIVERSITY OF NOTTINGHAM	HHMI International Early Career Scientist Instituto Gulbenkian de Ciência	11/06/2013	Oerias, Portugal	Scientific community (higher education, Research)	100	Europe
94	Organisation of Workshops	UNIVERSITA VITA-SALUTE SAN RAFFAELE	NABATIVI Summer School	01/07/2013	Milan, Italy	Scientific community (higher education, Research)	50	Europe
95	Oral presentation to a scientific event	POLYPHOR AG	NABATIVI Summer School	03/07/2013	Milan, Italy	Scientific community (higher education, Research)	50	Europe
96	Oral presentation to a scientific event	THE UNIVERSITY OF NOTTINGHAM	INTERNATIONAL SUMMER SCHOOL University of Granada	22/07/2013	Granada, Spain	Scientific community (higher education, Research)	40	Europe
97	Posters	THE UNIVERSITY OF NOTTINGHAM	14th International Conference on Pseudomonas	07/09/2013	Lausanne, Switzerland	Scientific community (higher education, Research)	400	Europe, USA
98	Oral presentation to a scientific event	POLYPHOR AG	IMAP 2013	24/09/2013	London, UK	Scientific community (higher education, Research)	150	Europe
99	Oral presentation to a scientific event	THE UNIVERSITY OF NOTTINGHAM	International seminar at the University of Jiangsu	06/10/2013	Jiangsu, China	Scientific community (higher education, Research)	300	Europe, Asia, USA
100	Posters	UNIVERSITA VITA-SALUTE SAN RAFFAELE	27th North-American Cystic Fibrosis Conference	17/10/2013	Salt Lake City, USA	Scientific community (higher education, Research)	1000	USA

Section B (Confidential[6] or public: confidential information to be marked clearly)
Part B1

TEMPLATE B1: LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS, ETC.					
Type of IP Rights[7]:	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Application reference(s) (e.g. EP123456)	Subject or title of application	Applicant (s) (as on the application)
None					

[6] Note to be confused with the "EU CONFIDENTIAL" classification for some security research projects.

[7] A drop down list allows choosing the type of IP rights: Patents, Trademarks, Registered designs, Utility models, Others.

Part B2

Type of Exploitable Foreground[8]	Description of exploitable foreground	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Exploitable product(s) or measure(s)	Sector(s) of application[9]	Timetable, commercial or any other use	Patents or other IPR exploitation (licences)	Owner & Other Beneficiary(s) involved
Commercial exploitation of R&D results General advancement of knowledge	5 selected P. aeruginosa targets for antibacterial development	Yes	Not determined	Targets inhibitors	Scientific research and development	2015	A patent is planned for 2014	University of Notthingham (owner), Università Vita e Salute, University of Zurich
Commercial exploitation of R&D results	POL7001	No	Not determined	antibacterial product	General medical practice activities	2018	Patented (WO 2007/079605 A2)	Polyphor
Commercial exploitation of R&D results	PNAs	Yes	Not determined	antibacterial product	General medical practice activities	2020	Patents are planned for 2014	University of Copenhagen (owner), University of Notthingham, Università Vita e Salute

Virulence and essential targets selected in the NABATIVI project are novel and have never been explored in *P. aeruginosa*. They will be exploited and characterize by University of Notthingham, Università Vita e Salute and University of Zurich in 2013 and 2014. IPR and the conditions for their distribution among partners were indicated in the Consortium Agreement. The outcomes will not only lead to an advanced knowledge on *P. aeruginosa* biology, but will also favour the discovery of inhibitory compounds that could be exploited as antimicrobials.

POL7001 belongs to a class of potent anti-*Pseudomonas* compounds produced by Polyphor and was investigated within the NABATIVI consortium as potential new anti- *P. aeruginosa* treatment in cystic fibrosis. High efficacy of this compound in murine models of lung infection was demonstrated. Studies will be implemented for further development in clinical trials. The compound is already patented (WO 2007/079605 A2). The impact of this discovery will be an innovative antibacterial treatment for *P. aeruginosa* infection in cystic fibrosis, but also its exploitation in other indications (sepsis, UTI, VAP).

[19] A drop down list allows choosing the type of foreground: General advancement of knowledge, Commercial exploitation of R&D results, Exploitation of R&D results via standards, exploitation of results through EU policies, exploitation of results through (social) innovation.

[9] A drop down list allows choosing the type sector (NACE nomenclature) : http://ec.europa.eu/competition/mergers/cases/index/nace_all.html

PNAs synthetized and validated in the NABATIVI project represent a new antibacterial strategy to fight *P. aeruginosa* infection. The targets inhibited by selected PNAs are both essential and virulence genes. Further ADMET and efficacy studies are necessary to evaluate their use in the clinic. IPR and the conditions for their distribution among partners were indicated in the Consortium Agreement and will potentially lead to patents. Whether these molecules may results to be highly efficacious in fighting *P. aeruginosa* infections is under investigation.

4.3 Report on societal implications

A General Information *(completed automatically when **Grant Agreement number** is entered.*	
Grant Agreement Number:	223670
Title of Project:	Novel Approaches to Bacterial Target Identification, Validation
Name and Title of Coordinator:	Alessandra Bragonzi, , PhD

B Ethics	
1. Did your project undergo an Ethics Review (and/or Screening)? • If Yes: have you described the progress of compliance with the relevant Ethics Review/Screening Requirements in the frame of the periodic/final project reports? Special Reminder: the progress of compliance with the Ethics Review/Screening Requirements should be described in the Period/Final Project Reports under the Section 3.2.2 *'Work Progress and Achievements'*	***Yes***
2. Please indicate whether your project involved any of the following issues (tick box) :	
RESEARCH ON HUMANS	
• Did the project involve children?	No
• Did the project involve patients?	No
• Did the project involve persons not able to give consent?	No
• Did the project involve adult healthy volunteers?	No
• Did the project involve Human genetic material?	No
• Did the project involve Human biological samples?	Yes
• Did the project involve Human data collection?	Yes
RESEARCH ON HUMAN EMBRYO/FOETUS	
• Did the project involve Human Embryos?	No
• Did the project involve Human Foetal Tissue / Cells?	No
• Did the project involve Human Embryonic Stem Cells (hESCs)?	No
• Did the project on human Embryonic Stem Cells involve cells in culture?	No
• Did the project on human Embryonic Stem Cells involve the derivation of cells from Embryos?	No
PRIVACY	
• Did the project involve processing of genetic information or personal data (eg. health, sexual lifestyle, ethnicity, political opinion, religious or philosophical conviction)?	No
• Did the project involve tracking the location or observation of people?	
RESEARCH ON ANIMALS	
• Did the project involve research on animals?	Yes
• Were those animals transgenic small laboratory animals?	Yes
• Were those animals transgenic farm animals?	No
• Were those animals cloned farm animals?	No
• Were those animals non-human primates?	No
RESEARCH INVOLVING DEVELOPING COUNTRIES	
• Did the project involve the use of local resources (genetic, animal, plant etc)?	**No**

• Was the project of benefit to local community (capacity building, access to healthcare, education etc)?	**No**
DUAL USE	
• Research having direct military use	NO
• Research having the potential for terrorist abuse	NO

C Workforce Statistics

3. Workforce statistics for the project: Please indicate in the table below the number of people who worked on the project (on a headcount basis).

Type of Position	Number of Women	Number of Men
Scientific Coordinator	1	0
Work package leaders	2	8
Experienced researchers (i.e. PhD holders)	12	23
PhD Students	8	3
Other	25	11

4. How many additional researchers (in companies and universities) were recruited specifically for this project?	**11**
Of which, indicate the number of men:	5

D Gender Aspects

5. Did you carry out specific Gender Equality Actions under the project?	X ○	Yes No

6. Which of the following actions did you carry out and how effective were they?

		Not at all effective				Very effective
X	Design and implement an equal opportunity policy	○	○	○	X	○
X	Set targets to achieve a gender balance in the workforce	○	○	○	X	○
X	Organise conferences and workshops on gender	○	○	○	X	○
X	Actions to improve work-life balance	○	○	○	X	○
○	Other:					

7. Was there a gender dimension associated with the research content – i.e. wherever people were the focus of the research as, for example, consumers, users, patients or in trials, was the issue of gender considered and addressed?

○ Yes- please specify

X No

E Synergies with Science Education

8. Did your project involve working with students and/or school pupils (e.g. open days, participation in science festivals and events, prizes/competitions or joint projects)?

○ Yes- please specify

X No

9. Did the project generate any science education material (e.g. kits, websites, explanatory booklets, DVDs)?

○ Yes- please specify

X No

F Interdisciplinarity

10. Which disciplines (see list below) are involved in your project?

X Main discipline[10]: Biological sciences

X Associated discipline[10]: Health sciences ○ Associated discipline[10]:

G Engaging with Civil society and policy makers

11a Did your project engage with societal actors beyond the research community? *(if 'No', go to Question 14)*	○ X	Yes No

11b If yes, did you engage with citizens (citizens' panels / juries) or organised civil society (NGOs, patients' groups etc.)?

○ No

[10] Insert number from list below (Frascati Manual).

- ○ Yes- in determining what research should be performed
- ○ Yes - in implementing the research
- ○ Yes, in communicating /disseminating / using the results of the project

11c In doing so, did your project involve actors whose role is mainly to organise the dialogue with citizens and organised civil society (e.g. professional mediator; communication company, science museums)?	○ ○	Yes No

12. Did you engage with government / public bodies or policy makers (including international organisations)

- X No
- ○ Yes- in framing the research agenda
- ○ Yes - in implementing the research agenda
- ○ Yes, in communicating /disseminating / using the results of the project

13a Will the project generate outputs (expertise or scientific advice) which could be used by policy makers?

- ○ Yes – as a **primary** objective (please indicate areas below- multiple answers possible)
- ○ Yes – as a **secondary** objective (please indicate areas below - multiple answer possible)
- X No

13b If Yes, in which fields?

Agriculture		Energy		Human rights	
Audiovisual and Media		Enlargement		Information Society	
Budget		Enterprise		Institutional affairs	
Competition		Environment		Internal Market	
Consumers		External Relations		Justice, freedom and security	
Culture		External Trade		Public Health	
Customs		Fisheries and Maritime Affairs		Regional Policy	
Development Economic and Monetary Affairs		Food Safety		Research and Innovation	
		Foreign and Security Policy		Space	
Education, Training, Youth		Fraud		Taxation	
Employment and Social Affairs		Humanitarian aid		Transport	

13c If Yes, at which level?

- ○ Local / regional levels
- ○ National level
- ○ European level
- ○ International level

H Use and dissemination

14. How many Articles were published/accepted for publication in peer-reviewed journals?	**28**
To how many of these is open access[11] provided?	**13**
How many of these are published in open access journals?	**8**
How many of these are published in open repositories?	**5**
To how many of these is open access not provided?	**15**
Please check all applicable reasons for not providing open access:	
❑ publisher's licensing agreement would not permit publishing in a repository ❑ no suitable repository available X no suitable open access journal available ❑ no funds available to publish in an open access journal ❑ lack of time and resources ❑ lack of information on open access ❑ other[12]: ……	

15. How many new patent applications ('priority filings') have been made? *("Technologically unique": multiple applications for the same invention in different jurisdictions should be counted as just one application of grant).*		**0**
16. Indicate how many of the following Intellectual Property Rights were applied for (give number in each box).	Trademark	**0**
	Registered design	**0**
	Other	**0**
17. How many spin-off companies were created / are planned as a direct result of the project?		**0**
Indicate the approximate number of additional jobs in these companies:		

18. Please indicate whether your project has a potential impact on employment, in comparison with the situation before your project:

❑	Increase in employment, or	❑	In small & medium-sized enterprises
❑	Safeguard employment, or	❑	In large companies
❑	Decrease in employment,	❑	None of the above / not relevant to the project
X	Difficult to estimate / not possible to quantify		

[11] Open Access is defined as free of charge access for anyone via Internet.
[12] For instance: classification for security project.

19. For your project partnership please estimate the employment effect resulting directly from your participation in Full Time Equivalent (*FTE = one person working fulltime for a year*) jobs:	*Indicate figure:*
Difficult to estimate / not possible to quantify	X

I Media and Communication to the general public

20. As part of the project, were any of the beneficiaries professionals in communication or media relations?

○ Yes X No

21. As part of the project, have any beneficiaries received professional media / communication training / advice to improve communication with the general public?

○ Yes X No

22 Which of the following have been used to communicate information about your project to the general public, or have resulted from your project?

❑	Press Release	❑	Coverage in specialist press
❑	Media briefing	❑	Coverage in general (non-specialist) press
❑	TV coverage / report	❑	Coverage in national press
❑	Radio coverage / report	❑	Coverage in international press
X	Brochures /posters / flyers	X	Website for the general public / internet
❑	DVD /Film /Multimedia	❑	Event targeting general public (festival, conference, exhibition, science café)

23 In which languages are the information products for the general public produced?

❑	Language of the coordinator	X	English
❑	Other language(s)		

***Question F-10*:** Classification of Scientific Disciplines according to the Frascati Manual 2002 (Proposed Standard Practice for Surveys on Research and Experimental Development, OECD 2002):

FIELDS OF SCIENCE AND TECHNOLOGY

1. NATURAL SCIENCES

1.1 Mathematics and computer sciences [mathematics and other allied fields: computer sciences and other allied subjects (software development only; hardware development should be classified in the engineering fields)]

1.2 Physical sciences (astronomy and space sciences, physics and other allied subjects)

1.3 Chemical sciences (chemistry, other allied subjects)

1.4 Earth and related environmental sciences (geology, geophysics, mineralogy, physical geography and other geosciences, meteorology and other atmospheric sciences including climatic research, oceanography, vulcanology, palaeoecology, other allied sciences)

1.5 Biological sciences (biology, botany, bacteriology, microbiology, zoology, entomology, genetics, biochemistry, biophysics, other allied sciences, excluding clinical and veterinary sciences)

2 ENGINEERING AND TECHNOLOGY

2.1 Civil engineering (architecture engineering, building science and engineering, construction engineering, municipal and structural engineering and other allied subjects)

2.2 Electrical engineering, electronics [electrical engineering, electronics, communication engineering and systems, computer engineering (hardware only) and other allied subjects]

2.3. Other engineering sciences (such as chemical, aeronautical and space, mechanical, metallurgical and materials engineering, and their specialised subdivisions; forest products; applied sciences such as geodesy, industrial chemistry, etc.; the science and technology of food production; specialised technologies of interdisciplinary fields, e.g. systems analysis, metallurgy, mining, textile technology and other applied subjects)

3. MEDICAL SCIENCES

3.1 Basic medicine (anatomy, cytology, physiology, genetics, pharmacy, pharmacology, toxicology, immunology and immunohaematology, clinical chemistry, clinical microbiology, pathology)

3.2 Clinical medicine (anaesthesiology, paediatrics, obstetrics and gynaecology, internal medicine, surgery, dentistry, neurology, psychiatry, radiology, therapeutics, otorhinolaryngology, ophthalmology)

3.3 Health sciences (public health services, social medicine, hygiene, nursing, epidemiology)

4. AGRICULTURAL SCIENCES

4.1 Agriculture, forestry, fisheries and allied sciences (agronomy, animal husbandry, fisheries, forestry, horticulture, other allied subjects)

4.2 Veterinary medicine

5. SOCIAL SCIENCES

5.1 Psychology

5.2 Economics

5.3 Educational sciences (education and training and other allied subjects)

5.4 Other social sciences [anthropology (social and cultural) and ethnology, demography, geography (human, economic and social), town and country planning, management, law, linguistics, political sciences, sociology, organisation and methods, miscellaneous social sciences and interdisciplinary , methodological and historical S1T activities relating to subjects in this group. Physical anthropology, physical geography and psychophysiology should normally be classified with the natural sciences].

6. HUMANITIES

6.1 History (history, prehistory and history, together with auxiliary historical disciplines such as archaeology, numismatics, palaeography, genealogy, etc.)

6.2 Languages and literature (ancient and modern)

6.3 Other humanities [philosophy (including the history of science and technology) arts, history of art, art criticism, painting, sculpture, musicology, dramatic art excluding artistic "research" of any kind, religion, theology, other fields and subjects pertaining to the humanities, methodological, historical and other S1T activities relating to the subjects in this group]