

Associazione Italiana Sindrome di Shwachman-Diamond (AISS)  
Via Pioveghetto 15, - 35136 Padova  
Tel - FAX +39 049 8736130  
E-mail: aiss@shwachman.it

## **Shwachman-Diamond Syndrome Italian Research Grant**

**Maximum Amount euro 10.000,00**

**Firm Deadline for Receipt of Applications: 31 December 2016**

Eligibility: Persons applying for these grants if not in a faculty position need to provide a declaration by a supervisor with a position in the department (not a training position) and with authority to hold an independent research grant.

Terms of Support: Support may be provided for one (1) year in an amount not to exceed E 10,000.

Indirect costs are permitted and are not to exceed 10% of the total costs.

The AISS will provide preference to those applications in which funds are used for supplies, equipment, technicians and other expenses and not for support of the salary of the PI or co-PIs.

Review: All applications will be reviewed by the AISS Scientific Committee (AISS-SC) or its designees.

Application: The application contains two sections.

Section 1, forms attached. The applicant and co-applicants must also include a current curriculum vitae.  
Section 2: Research Plan, divided as indicated below. Parts A through D should not exceed 6 pages, using a font no smaller than 10 point.

Part A. Specific aims

Part B. Significance and background

Part C. Preliminary studies

Part D. Experimental design and methods

Part E. References (not included in the 6 page limit)

Part F. Relevance of the research to Shwachman-Diamond Syndrome

Part G. For junior faculty separate letter from supervisor or department head confirming commitment to project, and to provision of space and facilities

Part K. If human subjects and animals are involved, a statement by the PI or supervisor overseeing human or animal studies is compulsory. If considered as necessary by the AISS-SC, more information about ethical committee study approval may be asked.

Submission by email to the AISS: aiss@shwachman.it

**1. Title of Proposal**

**Preclinical evaluation of the effect of Ataluren in restoring the expression of mutated SBDS protein in SDS cells**

**2. Applicant Information:**

Name:

Marco Cipolli

Title and Degree(s):

MD; Director of the Cystic Fibrosis Center, Ancona (Italy)

Work Address:

Cystic Fibrosis Center, Ospedali Riuniti, via Conca 71, 60020 Ancona

Phone: 071 5962035

Fax: 071 5962067

Email:

marco.cipolli@ospedaliriuniti.marche.it

**3. Applicant Curriculum Vitae:** beginning on the next page, with 2 page limit. This will form application pages 3 and 4.

- Born in Verona on the 29th of September 1959.
  - School-leaving examination in a classical high school – 1978.
  - Degree in Medicine and Surgery – 1985.
  - Specialisation in Paediatrics – 1990.
  - Specialisation in Gastroenterology and Digestive Endoscopy – 1994.
  - Attending Doctor at the Paediatric Ward of the Ospedale Civile in Verona from 16/12/1985 to 10/3/1987.
  - Scholarship Doctor at Veneto's Regional Centre for Cystic Fibrosis from 1/6/1988 to 30/10/1992.
  - Assistant Doctor at Veneto's Regional Centre for Cystic Fibrosis from 8/9/1993 to 29/12/1993.
  - Head of the Gastroenterological Clinic Service of the Cystic Fibrosis Centre of the Ospedale Civile Maggiore since 2/5/1997.
  - Director at Cystic Fibrosis Centre, University Hospital Ancona from 01 June 2016 to present
- version history 16/11/2016

- Member of the Italian Society for Gastroenterology and Paediatric Hepatology since 1996.
- Member of the Veneto Region Commission for Domiciliary Artificial Nutrition from 2/6/1998 (deliberation of the Regional Council n° 2036 del 2/6/1998) to 2009.
- Member of the Medical and Scientific Advisory Board (MSAB-US) of the Shwachman-Diamond Syndrome Intl since 2001. Chief of the MSAB-US from 2003 to 2005.
- Member of the Scientific Advisory Committee of the Italian Shwachman-Diamond Syndrome Association from 2004. Chief from 2007.
- Honorary observer in Gastroenterology at The Children Hospital at Westmead, Sydney, Australia from February to April 2002, from November to December 2005 and from February to March 2008.
- Teacher at the Paediatric Specialisation School at the University of Verona from 2003 to 2010.
- Member of the Scientific Committee of the European Cystic Fibrosis Society since 2008.

#### Expertises

- Statistical analysis of clinical trials.

Scuola Superiore di Oncologia e Scienze Biomediche. Genoa, 23-28 September 1991.

- Implementation and management of clinical trials.

Scuola Internazionale di Oncologia e Medicina Sperimentale. Rome, 15-20 November 1993.

- Survival analysis.

Corso di perfezionamento in Statistica Medica ed Epidemiologia Clinica. University of Verona. Verona, 1-2 July 1999.

#### Articles published on SDS from 2014

- Mercuri A, Cannata E, Perbellini O, Cugno C, Balter R, Zaccaron A, Tridello G, Pizzolo G, Bortoli MD, Krampera M, Cipolli M, Cesaro S. Immunophenotypic Analysis of Hematopoiesis in Patients suffering from Shwachman-Bodian-Diamond Syndrome. Eur J Haematol. 2014 Nov 17. doi: 10.1111/ejh.12490. [Epub ahead of print]

- Nacci L, Danesino C, Sainati L, Longoni D, Poli F, Cipolli M, Perobelli S, Nicolis E, Cannioto Z, Morini J, Valli R, Pasquali F, Minelli A. Absence of acquired copy number neutral loss of heterozygosity (CN-LOH) of chromosome 7 in a series of 10 patients with Shwachman-Diamond syndrome. *Br J Haematol*. 2014 May;165(4):573-5. doi: 10.1111/bjh.12767. Epub 2014 Feb 1
- Perobelli S., Alessandrini F., Zoccatelli G., Nicolis E., Beltramello A., Assael B.M., Cipolli M. Diffuse alterations in grey and white matter associated with cognitive impairment in Shwachman-Diamond syndrome: evidence from a multimodal approach. *Neuroimage Clin*. 2015 Feb 27;7:721-31. doi: 10.1016/j.nicl.2015.02.014. eCollection 2015.
- Ravera S, Dufour C, Cesaro S, Bottega R, Faleschini M, Cuccarolo P, Corsolini F, Usai C, Columbaro M, Cipolli M, Savoia A, Degan P, Cappelli E. Evaluation of energy metabolism and calcium homeostasis in cells affected by Shwachman-Diamond syndrome. *Sci Rep*. 2016 May 5;6:25441. doi:10.1038/srep25441.
- Minelli A, Nacci L, Valli R, Pietrocola G, Ramenghi U, Locatelli F, Brescia L, Nicolis E, Cipolli M, Danesino C. Structural variation in SBDS gene, with loss of exon 3, in two Shwachman-Diamond patients. *Blood Cells Mol Dis*. 2016 Sep;60:33-5. doi: 10.1016/j.bcnd.2016.06.007. Epub 2016 Jun 22.
- Nacci L, Valli R, Maria Pinto R, Zecca M, Cipolli M, Morini J, Cesaro S, Boveri E, Rosti V, Corti P, Ambroni M, Pasquali F, Danesino C, Maserati E, Minelli A. Parental origin of the deletion del(20q) in Shwachman-Diamond patients and loss of the paternally derived allele of the imprinted L3MBTL1 gene. *Genes Chromosomes Cancer*. 2016 Aug 24. doi: 10.1002/gcc.22401. [Epub ahead of print]
- Bezzerri V, Vella A, Calcaterra E, Finotti A, Gasparello J, Gambari R, Assael BM, Cipolli M, Sorio C. New insights into the Shwachman-Diamond Syndrome-related haematological disorder: hyper-activation of mTOR and STAT3 in leukocytes. *Sci Rep*. 2016 Sep 23;6:33165. doi: 10.1038/srep33165

**4. Applicant's Commitment as Investigator of the Project:**

I agree as the applicant to accept responsibility for the scientific management of this project as outlined in this application. I further agree to submit a report at the end of the granting period.

**5. Applicant's Affirmation:**

I certify that the investigations involving human subjects to be carried out in the application will have approval of the applicant's Institutional Ethical Committee

Approvals from the Institutional Ethical Committee must be included with the application.

**6. Research Results:**

Results of research may be made available to the public through appropriate scientific channels. All publications will bear the statement:



---

Signature of Applicant

28/12/2016  
Date

**7. Applicant's Institution Certification and Commitment:**

I certify that the statements herein and the Applicant's Affirmation are true, complete and accurate to the best of my knowledge and I agree to accept responsibility for the fiscal management of this project as outlined in this application. I further agree to commit this institution to comply with the Associazione Italiana Sindrome di Shwachman-Diamond (AISS) terms and conditions if a grant is awarded as a result of this application.

Name of Institution Official: Dott. Marco Cipolli

Title: Director of the Cystic Fibrosis Center, Ospedali Riuniti, Ancona

Address: via Conca 71, 60020-Ancona

Phone: 071 5962035

Fax: 071 5962067

E-mail Address: marco.cipolli@ospedaliriuniti.marche.it



---

Signature of Institution Official

28/12/2016  
Date

**ABSTRACT OF RESEARCH PLAN**

Within the space provided, summarize the long-term objectives, scientific aims and methodology of the proposal.

**TITLE: Preclinical evaluation of the effect of Ataluren in restoring the expression of mutated SBDS protein in SDS cells**

Data from the Italian SDS registry revealed that most of SDS patients (56%) carry the nonsense mutation 183-184 TA→TC. This mutation introduces a premature termination codon (PTC), resulting in the amino-acid change K62X. PTC leads to accelerated degradation of mRNA, a process known as nonsense-mediated decay (NMD). PTC can be endogenously overridden by alterations in the translation process, namely nonsense suppression, which allows the misreading of PTC by near-cognate tRNA. In the last decade, several PTC suppressor molecules have been developed, assuming that small molecules such as aminoglycosides promote PTC read-through. Among the PTC suppressors, Ataluren has been recently approved for the treatment of Duchenne muscular dystrophy and has been testing in clinical trials for the treatment of Cystic Fibrosis. Thus, we will test the effect of Ataluren in correcting SBDS 183-184 TA→TC mutation both in cell lines and primary bone marrow stem cells obtained from SDS patients. In order to address this issue, we will firstly evaluate the efficacy of Ataluren in restoring the full-length SBDS protein expression by western blot analysis and immunofluorescence. Secondly, we will assess the functional restoration of SBDS protein. In this regard, we will verify the capability of bone marrow stem cells to develop myeloid progenitors in the presence or in the absence of Ataluren. To address this point, we will perform colony assays, based on the capability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid medium in response to cytokine stimulation. The colonies formed will be enumerated and characterized according to their unique morphology. Again, as we recently reported that SDS patients present hyper-phosphorylation of mTOR in S2448 residue, we will test the effect of Ataluren-driven correction of SBDS expression in reducing the excessive mTOR activation. In order to test mTOR phosphorylation, we will perform Phospho-flow analysis in SDS lymphoblastoid cells using conjugated antibodies against the phosphorylated form (S2448) of mTOR. We also test mTOR activation using specific ELISA kits, recognizing the S2448 mTOR phosphorylation. Our preliminary results indicate that Ataluren is able to correct SBDS protein expression both in lymphoblastoid cells and in primary CD34+ bone marrow stem cells obtained from SDS patients. This finding represents the proof of concept of a novel therapeutic approach in SDS pathology. Since Ataluren has been already approved by EMA for the treatment of other rare genetic diseases, if results will confirm the efficacy of Ataluren in correcting the expression of SBDS in bone marrow stem cells and in restoring its function, this study might lead to a clinical trial within few years.

**BUDGET**

List below a budget by categories for the support. The review committee will carefully consider the appropriateness of your budget. It must be well defined, justified, and realistic to complete the work proposed. The first column defines the total expenses that are expected to be necessary to realistically complete the project. The second column indicates the expenses requested from the AISS. Applicants **will not** be penalized in funding considerations for requiring additional funds beyond what is requested from the Foundation(AISS); however, the true costs of the project must be acknowledged.[This and the section on page 1 re: Other Funding need to be consistent]

**EURO Amount Requested for:**

	<b>TOTAL COSTS REQUIRED TO COMPLETE PROJECT:</b>	<b>COSTS REQUESTED FROM AISS:(not to exceed E 10,000)</b>
Personnel (including fringe benefits):		
PI: Name: Marco Cipolli	0	0
Co-I Name: Valentino Bezzeri	10000	0
Additional personnel (identify role):		
Name:		
Equipment:	0	0
Supplies:	20000	7000
Other Expenses:	5000	3000
Indirect Costs (not to exceed 10% of total)	0	0
<b>TOTAL COSTS:</b>	<b>35000</b>	<b>10000</b>



**Justification:** Define and justify expenses in each category. Explain the role of each of the individuals named in the Personnel section. The justification must include an explanation of what each category contributes to the project. Also explain any marked differences between the first- and second-year expenses in a particular category. The AISS will provide preference to those applications in which funds are used for supplies, equipment, technicians and other expenses and not for support of the salary of the PI or co-PIs. The AISS-SC may ask for further expense details.

Personnel: Marco Cipolli will coordinate the entire project. He will follow patient recruitment and clinical sample withdrawals. Valentino Bezzeri will manage the laboratory issues, he will also carry out most of the experiments.

Supplies (7,000 euro): this budget will be intended for reagents including flow cytometry buffers and antibodies, ELISA Kits, Colony assay Kits, media for bone marrow stem cell cultures, cytokines and growth factors, gels and antibodies for Western Blot analysis, plastic tubes and consumables. Supplies indicated above will be essential to address the proposed aims of the project (detection of SBDS protein expression, assessment of mTOR activation, colony assays).

Other (3,000 euro): expenses for travels and results dissemination (conferences, seminars, posters, publications).

**Other Support for this Project:**

Applicants are allowed to receive funding from other sources for parts of the project not funded by the AISS. Please, list all other funding sources.

## Research Plan

### Part A. Specific aims

No specific therapies aimed to correct the basic defect of SDS have been developed so far. The PTC suppressor molecule Ataluren has been recently approved by EMA for the treatment of Duchenne muscular dystrophy. Since 56% of the Italian SDS patients carry the nonsense mutation 183-184 TA→TC, the overall aim of this project is to verify the effect of Ataluren in correcting the 183-184 TA→TC mutated SBDS protein expression and function both in cell lines and clinical samples obtained from SDS patients. These findings might lead to a future clinical trial based on the use of Ataluren in SDS patients carrying nonsense mutations.

*Aim 1: To verify the effect of Ataluren in correcting mutated SBDS protein expression in bone marrow stem cells.* Although Ataluren has been already proven to restore protein expression in nonsense mutated genes causing Duchenne muscular dystrophy and cystic fibrosis (Wilton S, 2007; Welch EM, 2007), it has also recently reported that Ataluren read-through efficiency is tissue-specific, because of the tissue-nonsense mutant transcript level (Thada V, 2016). Thus, we will verify the effect of Ataluren on SBDS protein expression restoration by western blot analysis in different bone marrow stem cells, including CD34+ stem cells and mesenchymal stem cells (MSCs).

*Aim 2: To investigate the effect of Ataluren on myeloid differentiation in bone marrow progenitors obtained from SDS patients.* SDS is characterized by bone marrow failure, associated with increased apoptosis of hematopoietic progenitors (Dror Y, 2001). Indeed, low percentage of CD34+ progenitors, as well as decreased percentage of granulocyte, have been found in SDS bone marrow samples (Mercuri A, 2015). It has been also recently reported that mice lacking *Sbds* gene expression show reduced myeloid lineage progression, which in turn causes neutropenia (Zambetti NA, 2015). Thus, restoring SBDS expression in human CD34+ stem cells should promote myeloid differentiation. In order to address this issue, we will perform colony assays, in the presence or in the absence of Ataluren, using CD34+ stem cells freshly isolated from bone marrow samples obtained from SDS patients. Again, we will assess the capability of Ataluren to restore the normal myeloid differentiation rate by flow cytometry assay, evaluating the expression of common myeloid biomarkers such as CD16 (myeloid marker), CD11b (granulocyte maturation marker) and CD45 (pan-leukocyte marker). We will also perform colony assays in order to check the capability of Ataluren-treated bone marrow hematopoietic progenitor stem cells to differentiate into myeloid progenitors.

*Aim 3: To check the phosphorylation of mTOR S2448 upon Ataluren treatment in SDS cells.* We recently reported that mTOR S2448 phosphorylation is strongly up-regulated in SDS leukocytes, including neutrophils (Bezzetti V, 2016). Notably, the excessive activation of mTOR, in particular the mTORC1 pathway, is associated with hematological malignancies such as AML. Thus, we will test the effect of Ataluren treatment on mTOR phosphorylation both in SDS EBV-immortalized B cell model and in bone marrow stem cells. To check mTOR phosphorylation in single-cell we will perform flow cytometry assays using conjugated antibody against S2448 mTOR phosphorylated form. Furthermore we will check total mTOR S2448 phosphorylation in lymphoblastoid cells and in bone marrow stem cells using specific ELISA recognizing phospho-mTOR.

### Part B. Significance and background

Shwachman-Diamond Syndrome (SDS) is a rare recessive autosomal disease caused by mutations in the Shwachman-Bodian-Diamond Syndrome gene (SBDS) encoding the homonymous protein SBDS. SDS is characterized by multiple-organ diseases, including hematological disorders, bone malformation, pancreas insufficiency, cognitive impairment.

Among the clinical issues observed in SDS patients, the bone marrow failure is the major cause of morbidity and mortality. Most of SDS patients suffer of severe neutropenia, which in turn is the main cause of lung infection during the early stages of life. SDS is also associated to myelodysplastic syndrome (MDS), with high risk of Acute Myeloid Leukemia (AML) progression. Indeed, 11% of SDS patients recorded in the French Severe Chronic Neutropenia Registry reported MDS or AML<sup>1</sup>. Researchers have attempted great efforts investigating the exact role of SBDS within the SDS pathogenesis in the last decade. Several reports show that SBDS is involved in the biogenesis of ribosomes, indicating SDS as a ribosomopathy. Recently, it has been proposed that SDS is a multitasking protein which proofreads the peptide-exit tunnel within the ribosome machinery, promotes the GTPase elongation factor-like 1 (ELF1) conformational switching and mediates 60S binding by regulating the eukaryotic initiation factor 6 (eIF6) releasing<sup>2</sup>. Loss of SBDS expression has also been associated with the increased expression of the mammalian Target of Rapamycin (mTOR)<sup>3; 4</sup>. Importantly, mTOR pathway has recently emerged as one of the best characterized central pathways hematopoietic stem cell function<sup>5</sup>. Since mTOR dysregulation has been associated with several forms of leukaemia<sup>6</sup>, this process might at least partially explain the increased risk of AML development observed in SDS patients. Most of SDS patients (74.4%) have been reported to carry two common mutations, namely 183-184TA→TC and 258+2T→C<sup>7</sup>. The dinucleotide alteration 183-184TA→TC is a nonsense mutation which introduces a premature termination codon (PTC) resulting in the amino-acid change K62X, whereas the 258+2T→C alteration causes a missense mutation disrupting the donor splice site of intron 2<sup>7</sup>. Notably, among the 120 Italian SDS patients enrolled into the Italian SDS Registry, 56.4% of subjects carry the 183-184TA→TC nonsense mutation in one allele (unpublished data), similarly to the previous study based on a Canadian cohort of SDS patients<sup>7</sup>. Moreover, likewise the Canadian cohort, no homozygotes for this mutation are present in the Italian registry. Interestingly, about 20% of Italian SDS patients carrying 183-184TA→TC mutation developed MDS or leukemia (unpublished data), doubling the percentage of malignant transformation which has been reported for SDS patients recorded in the French Severe Chronic Neutropenia Registry<sup>1</sup>. Interestingly, data from Italian SDS registry revealed that only patients carrying the 183-184TA→TC mutation have early developed MDS/AML. PTCs can be functionally overridden by endogenous alterations in the translation process through a phenomenon called nonsense suppression, which allows the misreading of PTC by near-cognate tRNA<sup>8</sup>. Nonsense suppression can also be sustained by small molecules, such as aminoglycosides<sup>9</sup>, which promote the read-through of PTCs<sup>10</sup>. Among the small molecules promoting nonsense suppression, Ataluren (PTC124; 3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid) is a hopeful orally bioavailable compound which does not present structural similarity to aminoglycoside antibiotics<sup>11</sup>. Ataluren has been already approved by European Medicines Agency (EMA) for the treatment of Duchenne muscular dystrophy<sup>12</sup> and has been testing in phase III clinical trial for the treatment of cystic fibrosis<sup>13</sup>. The molecular mechanisms which underlie Ataluren-dependent nonsense suppression have been recently reported in human cells and yeasts expressing multiple PTC reporter genes<sup>14</sup>. In this work, authors showed that Ataluren promotes insertion of nearcognate tRNAs at the nonsense codon site, resulting in amino acid replacements similar to those observed in endogenous read-through<sup>14</sup>.

This study may represent a real breakthrough towards a new therapeutic approach for most of SDS patients carrying nonsense mutation. Since Ataluren has been already approved by EMA for the treatment of Duchenne muscular dystrophy, this project could lead to a future clinical trial on SDS patients within few years.

### **Part C. Preliminary studies**

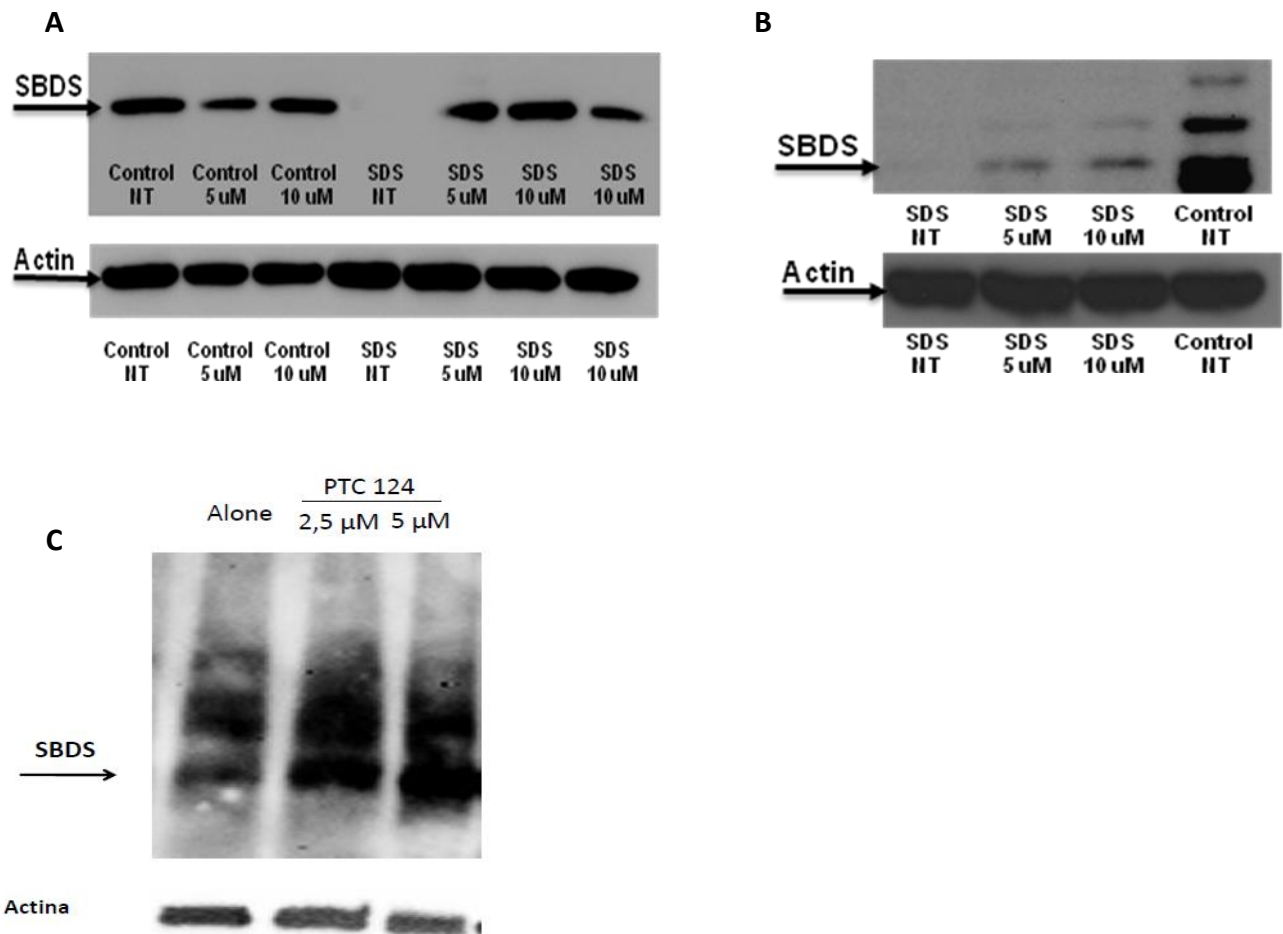
#### ***Ataluren restores SBDS full-length protein expression in SDS LCLs and BMSCs***

Ataluren has been already proven to be a small molecule able to act as nonsense suppressor in several cell types and pathologies<sup>14; 15; 16</sup>. However, other studies indicated a tissue-specific effect of nonsense suppression exerted by Ataluren, which might result in a weak response to the drug in some cases<sup>16;17</sup>. In order to investigate the read-through capability of Ataluren in hematological cells, we firstly treated LCLs obtained from SDS patients carrying the nonsense mutation 183-184TA→TC with 5-10  $\mu$ M Ataluren for 24 hours. Results indicated that 5  $\mu$ M Ataluren is sufficient to strongly restore full-length SBDS protein expression, as observed by western blot analysis (Fig. 1A). In order to exclude the effect of artifacts due to non specific antibody binding, we performed the same experiments staining another primary antibody recognizing SBDS. Results confirmed that SBDS expression was corrected in Ataluren-treated cells (Fig. 1B). Since Ataluren-mediated protein restoration in LCLs could be affected by signaling pathways activated by EBV infection in these cells, we confirmed the obtained result also in freshly isolated CD34+ BMSC and MSCs, which represents the main target of our study, being the bone marrow failure the main cause of mortality and morbidity in SDS. Again, results indicated an high improvement in protein expression restoration of SBDS upon 2.5-5  $\mu$ M Ataluren treatment for 24 hours (Fig. 1 C).

#### ***Ataluren-mediated SBDS correction improves differentiation of myeloid lineage from CD34+ BMSCs***

As observed in many other ribosomopathies, SDS patients present bone marrow failure. Over time, many patients undergo myelodysplastic syndrome, which in turn is associated with high risk to develop AML. Notably, data from the Italian SDS Registry show that about 20% of Italian patients carrying the nonsense 183-184TA→TC have developed MDS/leukemia. Moreover, SDS is characterized by severe neutropenia, further highlighting the need to improve the myeloid differentiation of bone marrow progenitors. Here, we tested the effect of 2.5-5  $\mu$ M Ataluren on BMSC myeloid differentiation using specific colony assays, which are already used as the golden standard of diagnostic tools regarding SDS-derived myelodysplasia follow up. CD34+ stem cells were incubated with different doses of Ataluren for 28 days and check of myeloid colony number was finally performed. Results indicated that 2.5  $\mu$ M Ataluren was sufficient to double the myeloid colony number compared to untreated condition, and, importantly, retrieving the number of colonies very nearly to the healthy untreated control condition (Fig. 2).

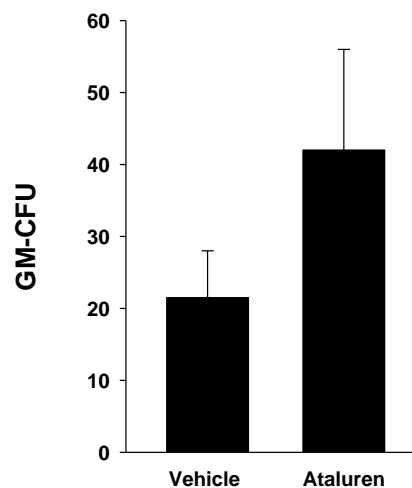
Figure 1



**Figure 1. Ataluren restores SBDS protein expression in SDS cells.** **A, B.** Lymphoblastoid cell lines obtained from healthy donors (control) or SDS patients carrying 183-184TA $\rightarrow$ TC mutation (SDS) were treated with 5-10  $\mu$ M Ataluren for 24 hours. Cells were lysed in the presence of protease inhibitors and total protein extracts were analyzed by wester blot using a primary rabbit polyclonal anti-SBDS antibody (**A**), or a mouse monoclonal anti-SBDS antibody (**B**). **C.** hematopoietic CD34+ stem cells were isolated from bone marrow of a SDS patient carrying 183-184TA $\rightarrow$ TC mutation and treated with 2.5-5  $\mu$ M Ataluren for 24 hours. Cells were lysed in the presence of protease inhibitors and total protein extracts were analyzed by wester blot using a primary mouse monoclonal anti-SBDS antibody.

Figure 2

version history 16/11/2016



**Figure 2. Ataluren improves myeloid differentiation in bone marrow stem cells withdrawn by SDS patients.**

CD34+ hematopoietic stem cells were freshly isolated from bone marrow samples of two SDS patients carrying the 183-184TA→TC mutation and colony assay was performed in the presence or in the absence (vehicle alone, DMSO) of 2.5  $\mu$ M Ataluren dissolved into the culture media. Cells were incubated for 28 days and myeloid (GM) colony forming units (CFU) were counted.

**Part D. Experimental design and methods****Patient recruitment**

Ten volunteer SDS patients will be recruited during the programmed Day Hospital visits for clinical evaluation at Cystic Fibrosis Center of Ancona. SDS patients will be included only if they carry the 183-184TA→TC mutation SDS mutation. Bone marrow samples (5 ml) will be obtained at the Unit of Pediatric Oncohematology, Azienda Ospedaliera Universitaria Ospedali Riuniti di Ancona, during the annual programmed hospitalization of SDS patients. Bone marrow sample (5 ml) of healthy sibling donor who undergo bone marrow harvest as donor for a related matched human-leukocyte-antigen transplant, will be used as healthy control. Samples and data will be obtained and used for analysis only after that informed consent will be signed according to the guidelines approved by the local Ethics Committee.

**Western Blot**

Cell proteins extracted from bone marrow stem cells will be separated on 11% SDS-PAGE, and electroblotted onto Immobilon P filters (Millipore, Billerica, MA) previously blocked with 5% BSA in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl) supplemented with 0.05% Tween-20 (TBS/T). The membranes will be probed with: i) anti-human SBDS rabbit polyclonal IgG antibody (amino acids 1 and 250 of SBDS, Abcam, Cambridge, MA, dilution 1:1500); ii) monoclonal anti- $\beta$ -Actin clone AC-15 (Sigma-Aldrich, diluted 1:2000) in 1% BSA TBS/T. Membranes will be incubated overnight at 4°C and after washes, membranes will be incubated with the secondary antibody, horseradish peroxidase-coupled anti-rabbit IgG (Sigma-Aldrich, dilution 1:15000), for 1 hour. Immunocomplexes will be detected using ECL Plus Western Blotting detection system (Amersham Biosciences, Little Chalfont, UK).

**Colony-forming assay**

CD34+ bone marrow stem cells will be isolated using immunomagnetic beads, plated in few mls and expanded at low density in StemSpan medium (Breda L, 2013) supplemented with StemSpan CC-100 cytokine cocktail, erythropoietin, dexamethasone and penicillin/streptomycin. Spontaneously differentiating and dead cells will be eliminated by density gradient centrifugation followed by medium replacement twice a week. Undifferentiated state will be assessed by flow cytometry, using C-kit, CD34, CD235a and Band3 antibodies. The sorted CD34+ hematopoietic cells will be plated in 35-mm culture dishes in methylcellulose media (MethoCult H4535, H4435, or MethoCult SFBIT H4236; StemCell Technologies, Vancouver, BC, Canada). The MethoCult H4435 (for both erythroid and myeloid lineages) is composed by EPO, G-CSF, GM-CSF, IL-3, and IL-6. At Day 14-21-28, colonies containing more than 50 cells will be counted and classified as granulocyte colony, monocyte/macrophage colony, erythroid blast colony, or mixture of granulocytes and erythroid colony, and colonies containing 20–50 cells of erythroid colony. The colonies will be counted by standard criteria in 60-mm gridded scoring dishes with cross marks (Stem Cell Technologies) under an inverted microscope. At this time, macroscopic red erythroid colonies will be readily distinguishable from purely granulopoietic colonies.

**Flow cytometry**

CD34+ bone marrow stem cells will be isolated using immunomagnetic beads, plated in few mls and expanded at low density in StemSpan medium (Breda L, 2013) supplemented with StemSpan CC-100 cytokine cocktail, erythropoietin, dexamethasone and penicillin/streptomycin. Spontaneously differentiating and dead cells will be eliminated by density gradient centrifugation followed by medium replacement twice a week. Undifferentiated state will be assessed by flow cytometry, using C-kit, CD34, CD235a and Band3 antibodies. CD34+ isolated cells will be incubated at 37 °C in the presence or in the absence of 2.5-5 µM Ataluren (PTC Therapeutics, South Plainfield, NJ) for 24, 48 or 72 hours. Cells will be stained with following fluoro-conjugated antibodies: anti-CD11b, anti-CD16, anti-CD45 (all supplied by Beckman-Coulter, Indianapolis, IN). CD34+ bone marrow cells and Lymphoblastoid cell lines obtained from SDS patients will be fixed and permeabilized with Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, CA), following the manufacturer's protocol. After permeabilization, cells will be washed once in flow buffer and stained with anti p-S2448-mTOR-PE or isotype control-PE conjugated antibodies for 30 minutes. Cells will be washed and acquired on a 10 colours, 3 laser (Blue Solid State Diode: 488nm, 22mW, Red Solid State Diode: 638nm, 25mW, Violet Solid State Diode: 405nm, 40mW), Navios flow cytometer (Beckman Coulter, Indianapolis, IN). All acquired data files will be analyzed using the "Navios" or Kaluza software, version 1.3 (Beckman Coulter, Indianapolis, IN). In order to check the effect of Ataluren in regulating myeloid differentiation in CD34+ bone marrow cells, CD45 versus SS gating strategy will be used to recovery hematopoietic progenitor differentiation stages, whereas CD45 positive region will be plotted on CD11b versus CD16 dotplot to check myeloid maturation.

**ELISA**

Lymphoblastoid cell lines and CD34+ bone marrow stem cells obtained from SDS patients will be treated in the presence or in the absence of increasing doses of Ataluren (0.1, 1, 2.5, 5, 10 µM) for 24 hours. The mTOR phosphorylation of S2448 will be detected by Pathscan phospho-mTOR ELISA (Cell Signaling Technology, Danvers, MA) following the manufacturer's protocol.

**Part E. References**

- <sup>1</sup> DONADIEU, J. et al. Analysis of risk factors for myelodysplasias, leukemias and death from infection among patients with congenital neutropenia. Experience of the French Severe Chronic Neutropenia Study Group. **Haematologica**, v. 90, n. 1, p. 45-53, Jan 2005. ISSN 1592-8721. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/15642668> >.
- <sup>2</sup> WEIS, F. et al. Mechanism of eIF6 release from the nascent 60S ribosomal subunit. **Nat Struct Mol Biol**, v. 22, n. 11, p. 914-9, Nov 2015. ISSN 1545-9985. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/26479198> >.
- <sup>3</sup> BEZZERRI, V. et al. New insights into the Shwachman-Diamond Syndrome-related haematological disorder: hyper-activation of mTOR and STAT3 in leukocytes. **Sci Rep**, v. 6, p. 33165, Sep 2016. ISSN 2045-2322. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/27658964> >.

- 4 RAVERA, S. et al. Evaluation of energy metabolism and calcium homeostasis in cells affected by Shwachman-Diamond syndrome. **Sci Rep**, v. 6, p. 25441, May 2016. ISSN 2045-2322. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/27146429> >.
- 5 ZHENG, P. et al. Cytopenia and autoimmune diseases: a vicious cycle fueled by mTOR dysregulation in hematopoietic stem cells. **J Autoimmun**, v. 41, p. 182-7, Mar 2013. ISSN 1095-9157. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/23375848> >.
- 6 HOSHII, T.; MATSUDA, S.; HIRAO, A. Pleiotropic roles of mTOR complexes in haemato-lymphopoiesis and leukemogenesis. **J Biochem**, v. 156, n. 2, p. 73-83, Aug 2014. ISSN 1756-2651. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/24962700> >.
- 7 BOOCOCK, G. R. et al. Mutations in SBDS are associated with Shwachman-Diamond syndrome. **Nat Genet**, v. 33, n. 1, p. 97-101, Jan 2003. ISSN 1061-4036. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/12496757> >.
- 8 BAXTER-ROSHEK, J. L.; PETROV, A. N.; DINMAN, J. D. Optimization of ribosome structure and function by rRNA base modification. **PLoS One**, v. 2, n. 1, p. e174, Jan 2007. ISSN 1932-6203. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/17245450> >.
- 9 BURKE, J. F.; MOGG, A. E. Suppression of a nonsense mutation in mammalian cells in vivo by the aminoglycoside antibiotics G-418 and paromomycin. **Nucleic Acids Res**, v. 13, n. 17, p. 6265-72, Sep 1985. ISSN 0305-1048. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/2995924> >.
- 10 MENDELL, J. T.; DIETZ, H. C. When the message goes awry: disease-producing mutations that influence mRNA content and performance. **Cell**, v. 107, n. 4, p. 411-4, Nov 2001. ISSN 0092-8674. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/11719181> >.
- 11 DENT, K. M. et al. Improved molecular diagnosis of dystrophinopathies in an unselected clinical cohort. **Am J Med Genet A**, v. 134, n. 3, p. 295-8, Apr 2005. ISSN 1552-4825. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/15723292> >.
- 12 RYAN, N. J.; LO, J. H. Delamanid: first global approval. **Drugs**, v. 74, n. 9, p. 1041-5, Jun 2014. ISSN 1179-1950. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/24923253> >.
- 13 KEREM, E. et al. Ataluren for the treatment of nonsense-mutation cystic fibrosis: a randomised, double-blind, placebo-controlled phase 3 trial. **Lancet Respir Med**, v. 2, n. 7, p. 539-47, Jul 2014. ISSN 2213-2619. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/24836205> >.
- 14 ROY, B. et al. Ataluren stimulates ribosomal selection of near-cognate tRNAs to promote nonsense suppression. **Proc Natl Acad Sci U S A**, v. 113, n. 44, p. 12508-12513, Nov 2016.



- ISSN 1091-6490. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/27702906> >.
- <sup>15</sup> YU, H. et al. Comparison of read-through effects of aminoglycosides and PTC124 on rescuing nonsense mutations of HERG gene associated with long QT syndrome. **Int J Mol Med**, v. 33, n. 3, p. 729-35, Mar 2014. ISSN 1791-244X. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/24366185> >.
- <sup>16</sup> THADA, V. et al. Tissue-specific variation in nonsense mutant transcript level and drug-induced read-through efficiency in the Cln1(R151X) mouse model of INCL. **J Cell Mol Med**, v. 20, n. 2, p. 381-5, Feb 2016. ISSN 1582-4934. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/26648046> >.
- <sup>17</sup> MCELROY, S. P. et al. A lack of premature termination codon read-through efficacy of PTC124 (Ataluren) in a diverse array of reporter assays. **PLoS Biol**, v. 11, n. 6, p. e1001593, 2013. ISSN 1545-7885. Disponível em: < <https://www.ncbi.nlm.nih.gov/pubmed/23824517> >.

#### **Part F. Relevance of the research to Shwachman-Diamond Syndrome**

Shwachman-Diamond Syndrome (SDS) is a neglected rare inherited disease affecting 1/168,000 newborns in Italy with a mean of 3.0 new cases/year. Since no specific therapy has been developed so far, the goal of this project is to investigate the efficacy of Ataluren, a nonsense mutation suppressor molecule which has been already approved by EMA for the treatment of Duchenne muscular dystrophy, in correcting SBDS protein expression and function in SDS patients carrying the common nonsense mutation 183-184TA→TC. This should be the first attempt to develop a real specific therapy for SDS patients.