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: 1st February 2018

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

ELUCIDATION OF ABERRANT SBDS SPLICING MECHANISMS TO DESIGN RNA-THERAPEUTICS FOR SHWACHMAN-DIAMOND SYNDROME

2**. Applicant Information:**

Name: Dario Balestra

Title and Degree(s): PhD

Work Address: University of Ferrara, Department of Life Sciences and Biotechnology, Via Fossato di Mortara 74, 44121, Ferrara.

Phone: 0532974485

Fax:

Email: blsdra@unife.it

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

Born in Ferrara on the 14th March 1983

EDUCATION

2012 PhD. Research doctorate in Biochemistry, Molecular Biology and Biotechnologies
2007 Master Degree in Biomolecular and Cellular Sciences. 110/110 magna cum laude
2005 Bachelor Degree in Biomolecular and Cellular Sciences. 110/110 magna cum laude

PROFESSIONAL EXPERIENCE

2012 - present Postdoctoral fellow
2014 - present Professor of the Course of "Recombinant Technologies" course in the BSc (Biological Sciences) at University of Ferrara.
2010-2011 Research Scholar at The Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, USA
2009-2011 PhD student

PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED JOURNALS

-Co-author (D. Balestra, F. Bernardi) of book chapter "Gene therapy for hemorrhagic and thrombotic diseases", into the book "Hemorrhagic and thrombotic diseases", Castaman-Falanga, edit Piccin. 2018
-Scalet D, Sacchetto C, Bernardi F, Pinotti M, van de Graaf S F.J and Balestra D. The somatic FAH c.1061C>A change counteracts the frequent FAH c.1062+5g>a mutation and permits U1snRNA-based splicing correction. J Hum Genet. 2018 Accepted
-Ferrarese M, Testa MF, Balestra D, Bernardi F, Pinotti M, Branchini A. Secretion of wild-type factor IX upon readthrough over F9 pre-peptide nonsense mutations causing hemophilia B. Hum Mutat. 2018 Feb 1. doi: 10.1002/humu.23404.
-Scalet D, Balestra D, Rohban S, Bovolenta M, Perrone D, Bernardi F, Campaner S, Pinotti M. Exploring Splicing-Switching Molecules For Seckel Syndrome Therapy. Biochim Biophys Acta. 2016 Sep 14. pii: S0925-4439(16)30229-0.
-Balestra D, Scalet D, Pagani F, Malgorzata ER, Rosella M, Bernardi F, Pinotti M. An Exon-Specific U1snRNA Induces A Robust Factor IX Activity In Mice Expressing Multiple Human FIX Splicing Mutants. Mol Ther Nucleic Acids. 2016 Oct 4;5(10):e370. doi: 10.1038/mtna.2016.77.
-Tajnik M, Rogalska M.E., Bussani E, Barbon E, Balestra D, Pinotti M, Pagani F. Molecular Basis and Therapeutic Strategies to Rescue Factor IX Variants That Affect Splicing and Protein Function. PLOS Genetics. 2016 May 26; 12(5):e1006082.
-Morciano G, Giorgi C, Balestra D, Marchi S, Perrone D, Pinotti M, Pinton P. Mcl-1 involvement in mitochondrial dynamics is associated with apoptotic cell death. Mol Biol Cell. 2015 Nov 4. pii: mbc.E15-01-0028.
-Balestra D, Barbon E, Scalet D, Cavallari N, Perrone D, Zanibellato S, Bernardi F, Pinotti M. Regulation of a strong F9 cryptic 5'ss by intrinsicelements and by combination of tailored U1snRNAs with antisense oligonucleotides. Hum Mol Genet. 2015 Sep 1;24(17):4809-16. doi: 10.1093/hmg/ddv205.
-Balestra D, Faella A, Margaritis P, Cavallari N, Pagani F, Bernardi F, Arruda VR, Pinotti M. An engineered U1 small nuclear RNA rescues splicing defective coagulation F7 gene expression in mice. J Thromb Haemost. 2014 Feb;12(2):177-85.
-Cavallari N, Balestra D, Branchini A, Maestri I, Chuamsunrit A, Sasanakul W, Mariani G, Pagani F, Bernardi F, Pinotti M. Activation of a cryptic splice site in a potentially lethal coagulation defect accounts for a functional protein variant. Biochim Biophys Acta. 2012 Jul;1822(7):1109-13. doi: 10.1016/j.bbadis.2012.03.001.
-Fernandez Alanis E, Pinotti M, Dal Mas A, Balestra D, Cavallari N, Rogalska ME, Bernardi F, Pagani F. An exon-specific U1 small nuclear RNA (snRNA) strategy to correct splicing defects. Hum Mol Genet. 2012 Jun 1;21(11):2389-98. doi: 10.1093/hmg/dds045.
-Pinotti M, Balestra D, Rizzotto L, Maestri I, Pagani F, Bernardi F. Rescue of coagulation factor VII function by the U1+5A snRNA. Blood. 2009 Jun 18;113(25):6461-4. doi: 10.1182/blood-2009-03-207613.
-Pinotti M, Rizzotto L, Balestra D, Lewandowska MA, Cavallari N, Marchetti G, Bernardi F, Pagani F. U1-snRNA-mediated rescue of mRNA processing in severe factor VII deficiency. Blood. 2008 Mar 1;111(5):2681-4.

AWARDS

-Young Investigator Award during the XXV Congress of the International Society on Thrombosis and Haemostasis (ISTH). Toronto (CDN). June 20th – 25th 2015

-"Best Abstract" Award during the SISET XXIII National Congress (Italian Society for the Study of Haemostasis and Thrombosis), helded on 6th- 9th November 2014, Milan (ITA)

-Calogero Vullo Award during the 57th National Meeting of the Italian Society of Biochemistry and Molecular Biology (SIB), September 18th-20th 2013, Ferrara (ITA).

-Young Invertigator Award during the XXIV Congress of the International Society on Thrombosis and Haemostasis (ISTH). Amsterdam (NL). June 29th – July 4th 2013

-"Best PhD thesis 2012 in Biochemistry, Molecular Biology and Biotechnologies" award by IUSS-Ferrara 1391 (The University Institute for Higher Studies), University of Ferrara.

-"Best of the Best oral comunication" award during the XXII National SISET Congress (Italian Society of Trombosis and Hemostasis). Vicenza (ITA) , 4th – 6th October 2012.

SCIENTIFIC COLLABORATOR/FOUNDED PROJECT

- 2017- 2019. Bayer Hemophilia Awards Program. Early Career Investigator Award

- 2014-2017. Telethon Grant: Development of a RNA-based therapeutic approach for Hemophilia B caused by exon-skipping mutations. Collaborator

- 2014-2016. Insight Basic Research Grant: Altered mRNA processing and FVIII biosynthesis/function as determinants of phenotype variability in the frequent Arg2016Trp Haemophilia A patients. Bando competitivo supportato da Novo Nordisk (hiip://www.access-to-insight.com/grant_basic). Collaborator

- 2009-2013. Telethon Grant: RNA−based therapeutic approaches for blood coagulation factor deficiencies caused by splicing mutations. Collaborator

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

2/03/2018
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: Prof. Roberto Gambari

Title: Director of the Department of Life Sciences and Biotechnology of the University of Ferrara

Address: University of Ferrara, Department of Life Sciences and Biotechnology, Via Fossato di Mortara 74, 44121, Ferrara.

Phone: 0532 974443

Fax: 0532 974500

E-mail Address: roberto.gambari@unife.it

Signature of Institution Official

2-03-2018
Date

ABSTRACT OF RESEARCH PLAN

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

TITLE: ELUCIDATION OF ABERRANT SBDS SPLICING MECHANISMS TO DESIGN RNA-THERAPEUTICS FOR SHWACHMAN-DIAMOND SYNDROME

MOST OF SDS PATIENTS (74.4%) HAVE BEEN REPORTED TO CARRY TWO COMMON MUTATIONS, NAMELY C.183-184TA/TC AND C.258+2T>C, ARISING FROM A GENE CONVERSION EVENT. THE C.258+2T>C ALTERATION HAVE BEEN FOUND IN ~90% OF SDS PATIENTS AND CAUSES THE DISRUPTION OF THE DONOR SPLICE SITE (5'SS) OF INTRON 2, RESULTING IN AN 8-BP DELETION CONSISTENT WITH USE OF AN UPSTREAM CRYPTIC SPLICE DONOR SITE AT POSITION C.251-252 AND LEADING TO PREMATURE TERMINATION OF THE ENCODED PROTEIN BY FRAMESHIFT. THE IDENTIFICATION OF C.258+2T>C HOMOZYGOTES SDS PATIENTS AND THE FINDING THAT SBDS KNOCK-OUT MICE RESULTED IN EARLY EMBRYONIC LETHALITY, SUGGEST THAT TRACE LEVELS OF CORRECT TRANSCRIPTS SHOULD ARISE ALSO IN THE PRESENCE OF THE C.258+2T>C MUTATION, AN ASPECT NEVER INVESTIGATED BEFORE. MODULATION OF RNA FOR THERAPEUTIC PURPOSES ATTRACTED GREAT ATTENTION IN THE LAST DECADES AND LED TO THE IDENTIFICATION OF TWO BASIC RNA-BASED CORRECTION APPROACHES THAT EXPLOIT I) MODIFIED U1 SMALL NUCLEAR RNA (U1SNRNA), PROMOTING EXON DEFINITION AND II) ANTISENSE MOLECULES (AON), MASKING CRYPTIC SPLICE SITES OR REGULATORY ELEMENTS. IN THIS PROJECT WE AIM AT TO ELUCIDATE THE MOLECULAR MECHANISMS UNDERLYING SDS CAUSED BY THE C.258+2T>C SPLICING MUTATION, AND DESIGN AND EVALUATE THE ABILITY OF MODIFIED U1/AON IN RESCUING SBDS SPLICING. TO ADDRESS THESE POINTS, WE WILL EVALUATE SPLICING PATTERN OF CELLS (HEK AND HUH7) TRANSIENTLY TRANSFECTED WITH SBDS MINIGENE (WILD TYPE OR MUTATED) ALONE OR IN COMBINATION WITH AON (SCANNING ALL SBDS EXON 2) TO IDENTIFY SPLICING REGULATORY ELEMENTS REGULATING THE USAGE OF THE CRYPTIC 5'SS. SPLICING WILL BE ANALIZED BY RT-PCR AND CAPILLARY ELECTROPHORESIS. PARTICULAR ATTENTION WILL BE GIVEN TO THE DETERMINATION OF RESIDUAL LEVELS OF SBDS EXPRESSION BY QPCR. THESE FINDINGS WILL BE ALSO EVALUATED IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) OF SDS PATIENTS CARRYING THE C.258+2T>C MUTATION. IN THE ATTEMPT TO RESCUE SBDS SPLICING, WE WILL EXPLOIT A PANEL OF MODIFIED U1-RNA DESIGNED TO TARGET THE MUTATED 5'SS OR DOWNSTREAM INTRONIC SEQUENCES OR AON TO EITHER MASK THE IDENTIFIED CRYPTIC 5'SS OR SPLICING REGULATORY ELEMENTS. THE ABILITY OF U1RNA AND AON TO RESCUE SPLICING WILL BE EVALUATED BY CO-TRANSFECTION ASSAYS WITH MINIGENES FOLLOWED BY QUALITATIVE (RT-PCR, CAPILLARY ELECTROPHORESIS, SEQUENCING) AND QUANTITATIVE (REAL-TIME RT-PCR) RNA EVALUATION. WHEN AVAILABLE, THE CORRECTION WILL BE EVALUATED DIRECTLY IN SDS PATIENTS' LEUKOCYTES. MOREOVER, COMBINATION OF MODIFIED U1S-RNA AND AON WILL BE ALSO TESTED TO ASSESS THEIR SYNERGISTIC EFFECTS. IF SUCCESSFUL, THIS STUDY MAY REPRESENT A REAL BREAKTHROUGH TOWARDS A NEW THERAPEUTIC APPROACH FOR MOST OF SDS PATIENTS CARRYING THE COMMON C.258+2T>C MUTATION.

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:

	TOTAL COSTS REQUIRED TO COMPLETE PROJECT:	**COSTS REQUESTED FROM AISS**:(not to exceed E 10,000)
Personnel (including fringe benefits): PI: Name: Dario Balestra Co-I Name: Mirko Pinotti Additional personnel (identify role): Name:	 10000 0	 0 0
Equipment:	0	0
Supplies:	10000	9000
Other Expenses:	5000	1000
Indirect Costs (not to exceed 10% of total)	0	0
TOTAL COSTS:	25000	10000

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: Dario Balestra will coordinate the entire project and will carry out most of the experiments. The activities will be conducted in strict collaboration with Mirko Pinotti, leading the laboratory at the University of Ferrara, where the PI grew up. In accordance with the application rules, we are not requesting financial support for the PI salary.

Supplies (9,000 euro): this budget will be intended for reagents PCR, capillary electrophoresis, real time PCR, RNA extraction, RNA retro-transcription, cloning, media for cell cultures, plastic tubes and consumables. Supplies indicated above will be essential to address the proposed aims of the project (detection of SBDS trace levels, evaluation of splicing regulatory elements, assessment of RNA-therapeutics).

Other (1,000 euro): shipping costs and expenses for dissemination of results (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

Potential therapeutic strategies acting on mRNA processing and able to rescue proper splicing impaired by disease-causing mutations provided promising results both in vitro and in vivo models of human disorders (Fernandez 2012, Balestra 2014, Nizzardo 2015, Dal Mas 2015, Balestra 2016, Tajnik 2016, Rogalska 2016, Scalet 2017). In this pioneer project we propose to apply these RNA-based strategies for the development of a therapy for Shwachman-Diamond Syndrome (SDS), currently without cure. The idea stems from the observation that 145/158 (92%) of SDS patients carry a splicing mutation (c.258+2t>c) (Boocock 2003). Studies in patients showed that the mutation leads to an 8-bp deletion consistent with the usage of an upstream cryptic donor splice site at position 251-252 and causing a frameshift and thus premature translation termination.

The main aim of the present proposal is to elucidate the molecular mechanisms leading to aberrant splicing pattern of SBDS caused by the frequent c.258+2t>c splicing mutation and to exploit them to design correction strategies that might lay the foundation for the development of therapy for SDS.

More specifically, we aim:

1. to elucidate the molecular mechanisms underlying SDS caused by the c.258+2t>c splicing mutation. Particular attention will be given to the identification of splicing regulatory elements located within SBDS exon 2 or in the proximal intronic sequences. The presence of Exonic or Intronic Splicing Enhancer (ESE or ISE) or silencers (ESS or ISS), which helps designing correction strategies, will be initially evaluated by antisense oligonucleotides (AON) masking target sequences, and their validation will be carried out through small deletion scanning.
2. to restore SBDS splicing by engineered version of the spliceosomal U1snRNAs, forcing the usage of the defective donor splice site, or antisense oligonucleotide (AON), either masking the cryptic donor splice site or negative regulatory elements, whose effect will be evaluated in cellular model of SDS and in patients' leucocytes.

The overall scope is to provide the proof-of-principle that the frequent c.258+2t>c splicing mutation can be rescued by RNA therapeutics, which will boost further investigations aimed at developing a therapy for SDS.

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 (Boocock

2003). SDS is characterized by multiple-organ diseases, including hematological disorders, bone malformation, pancreas insufficiency, cognitive impairment (Cipolli 2001). Among the clinical issues observed in SDS patients, the bone marrow failure is the major cause of morbidity and mortality. Neutropenia is the most common hematopoietic abnormality in almost all patients with SDS, resulting in high susceptibility to infection in early life. SDS is also associated to myelodyspalstic syndrome (MDS), with high risk of Acute Myeloid Leukemia (AML) progression. Patients with SDS report also malabsorption and malnutritions due to pancreatic insufficiency and deficiency of vitamin D and vitamin K, as well as delayed puberty and recurrent infections, which in turn increase the risk to develop osteoporosis (Toiviainen 2007). Most patients show different degrees of cognitive impairment (Kerr 2010), which represent a real drawback for independence and socialization (Perobelli 2012). Great efforts have been made to investigate the exact role of SBDS within the SDS pathogenesis in the last decade. SDS can be considered as a ribosomopathy, since different studies showed that SBDS is involved in the biogenesis of ribosomes. In particular, 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 (Weis 2015).

Most of SDS patients (74.4%) have been reported to carry two common mutations, namely the c.183-184TA/TC and the c.258+2t>c changes, arising from a gene conversion event from a pseudogene carrying both nucleotide changes (Boocock 2003). The large majority carry the c.258+2t>c alteration, which causes the disruption of the donor splice site (5'ss) of intron 2 and leads to the usage of an upstream cryptic splice donor site at position c.251-252. The resulting 8-bp deletion causes frameshift and premature termination of translation, and predict the synthesis of a truncated protein. It is worth nothing that SBDS knock-out mice resulted in early embryonic lethality (Zhang 2006, Finch 2011), thus indicating that a residual expression of SBDS is essential for survival. Interestingly, although the c.258+2t>c change occurs within the highly conserved dinucleotide of donor splice site and thus is considered as null mutation with no traces of correct transcripts, the presence of patients homozygotyes for this mutation could indicate that the c.258+2t>c alteration is associated to trace levels of SBDS, an aspect never investigated so far.

Modulation of RNA splicing for therapeutic purposes attracted great attention in the last decades. In particular, the extensive work in the field led to the identification of two basic RNA-based correction approaches that exploit i) modified U1 small nuclear RNA (U1snRNA), promoting exon definition and ii) antisense molecules, masking cryptic splice sites or regulatory elements.

i) In the earliest splicing step, the U1snRNA mediates the recognition of the donor splice site (5'ss) by the ribonucleoprotein U1snRNP (Horowitz 1994). Studies in various human disease cellular models indicated that

engineered U1snRNAs can rescue aberrant splicing caused by mutations at 5'ss (Fernandez 2012, Balestra 2014, Nizzardo 2015, Dal Mas 2015, Balestra 2016, Tajnik 2016, Rogalska 2016, Scalet 2017). Our group has successfully exploited U1snRNAs in cellular and animal models of coagulation factor deficiencies (FVII deficiency; Hemophilia B) as well as of other inherited diseases. In the model of Hemophilia B, we developed U1snRNAs targeting intronic sequences downstream of the defective exon (Exon-Specific U1snRNA, ExSpeU1), and showed in cellular models that a unique ExSpeU1 restores FIX function impaired by different mutations at the 5'ss but also at the acceptor (3'ss) splice site (Fernandez 2012). Very recently, the proof-of-principle has been also provided in animal models (Balestra 2016). Of great importance, ExSpeU1s bind to non-conserved intronic sequences significantly reduces risk of off-target events.

ii) An alternative approach is to interfere with splicing regulatory elements on pre-mRNA through antisense oligonucleotides (AON) (Wood 2007; Du 2009). AON have been successfully tested in vitro and in vivo for different human diseases (Scaffidi 2005) such as β-thalassemia (Suwanmanee 2002) or FV/FIX deficiency (Nuzzo 2013; Balestra 2015) to mask cryptic sites, to skip defective exons in Duchenne Muscular Dystrophy (Denti 2006), or, most importantly for our purposes, to promote exon inclusion in SMN2 mRNA in Spinal Muscular Atrophy (Hua 2008).

Altogether, these information lay the ground complementary approaches (ExSpeU1 and AONs) for the development of RNA-based therapies for SDS caused by the common c.258+2t>c splicing mutation, which will stem from the dissection of the pathogenic aberrant splicing mechanisms. It is worth noting that ExSpeU1 can be systemically delivered by adeno-associated virus vectors (AAV) to achieve a life-long therapeutic effect with one injection, whereas AON could ensure a prolonged effects (several weeks) and applied to patients non eligible for AAV injection (pre-existing anti-AAV antibodies). Moreover, splicing-switching molecules counteracting the effect of splicing mutations have the invaluable advantage of maintaining gene regulation in physiological tissues, and, by exploiting small cassettes, to overcome limitations related to vector-mediated delivery of large genes.

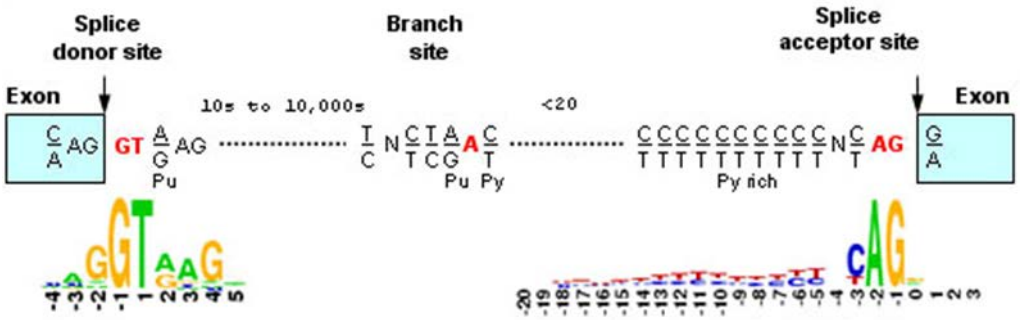
This study may represent a real breakthrough towards a new therapeutic approach for most of SDS patients carrying the common c.258+2t>c mutation.

Part C. Preliminary studies

As stated above this is the first attempt to provide a therapeutic strategy for SDS through intervention at the mRNA levels. Since this is a very pioneer project we did not produce any preliminary data on SBDS gene, and for this reason we are asking this initial financial support.

However, our extensive studies in the coagulation field, and particularly the investigation of mutations with features similar to that of the *SBDS* c.258+2t>c mutation provide us with feasibility elements that encourages

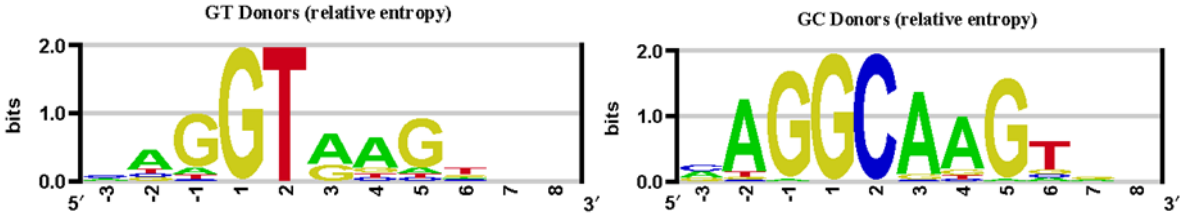
our attempt. Please find below the main elements of our rationale together with the data from other gene contexts.



Consensus sequences of introns of complex eukaryotes

The 5'ss is a short sequence (9 base pairs) defining the exon-intron boundary. It is composed by the last three nucleotides of exon and the first six nucleotides of the intron. The splice donor site includes an almost invariant sequence GU at the 5' end of the intron, within a larger, less highly conserved region.

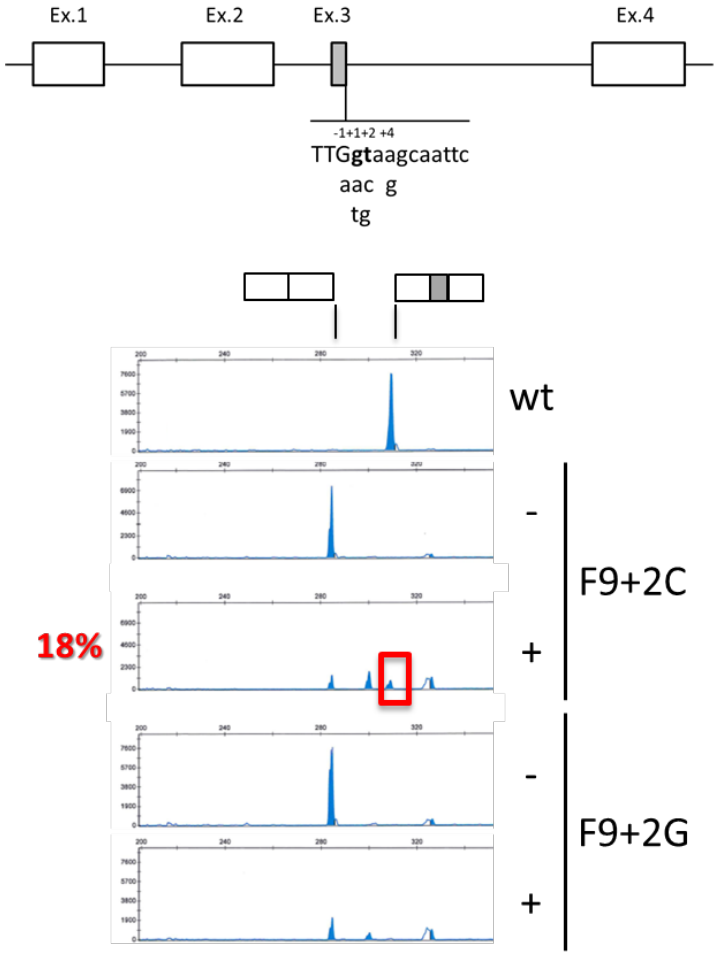
Mutations occurring in the highly conserved GT positions of the donor splice site (nucleotides +1 and +2 of the intron) are widely considered as "null" mutations, since no correct transcripts are expected at all. However, the observation that ~0.3% of introns has a non-canonical donor splice site in which the dinucleotide GC occurs instead of the GT, open the intriguing hypothesis that also splicing mutations as +2t>c could be associated to trace level of correct transcripts, justified also by the presence of homozygotes SDS patients with the c.258+2t>c variant.



Comparison between the common donor splice sites (left) and non-canonical (right) 5'ss

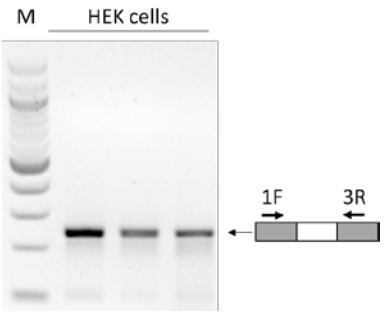
Concerning the ability of RNA-therapeutics, such as modified U1snRNA and/or AON, of rescuing splicing mutations, actually in literature there are no data showing the possibility to rescue these mutations. However, our recent data showed that, in some exon context, also mutations +2t>c can be rescued. In fact, in our cellular model of Hemophilia B (HB), various patients have been discovered with mutations at c.277+2t>c/g, all showing a very severe phenotype. Notably, the presence of both changes at the same

position offered the opportunity to assess the hypothesis that the +2t>c can be rescued by therapeutic strategies acting at RNA level. A therapeutic approach based on modified U1snRNA showed that the variant +2t>c can be efficiently rescued, with the appearance (~18%) of correct transcripts.



These data provide the first insight into the ability of targeting and rescuing the severest as well as the most common splicing mutations occurring at the conserved GT dinucleotide, which are associated to null conditions.

In this project we are proposing to characterize the molecular mechanisms leading to aberrant splicing by transfecting human embryonic kidney cells (HEK) with SBDS minigenes. Since the splicing process is cell-dependent, the cellular model in which we will investigate the effect and the rescue of the SBDS c.258+2t>c mutation plays a crucial role. Therefore, we have investigated the splicing pattern of SBDS in those cells to evaluate the presence of correctly spliced transcripts. As observed below, SBDS is expressed in HEK cells and exon 2 is correctly spliced, thus indicating that HEK cells represents a good cellular model in which perform our studies on SBDS splicing processing.



Expression of SBDS in HEK cells

Part D. Experimental design and methods

In this project, we propose to characterize the molecular mechanisms leading to aberrant splicing in the presence of the most frequent c.258+2t>c mutation and to identify modified U1snRNAs and/or AONs able to restore proper exon definition.

In particular, this one-year project will be articulated in the following work packages:

WP1: Identification and characterization of Splicing Regulatory Elements

WP2: Development of RNA-Based strategies

WP1:IDENTIFICATION AND CHARACTERIZATION OF SPLICING REGULATORY ELEMENTS

WP1.1_CREATION AND VALIDATION OF MINIGENES:

Expression of minigenes represents a well-established strategy for the characterization of splicing mechanisms in vitro and for testing the effects of new therapeutics acting at RNA level. Minigenes will be prepared by PCR amplification of SBDS exon 2 and the surrounding intronic sequences and cloning in the exon trapping plasmid pTB (Fernandez 2012). Briefly, pTB represents the basic hybrid minigene construct and consists of a modified version of the alpha-globin-fibronectin EDB minigene. The genomic cassette under investigation, either wild type or carrying the mutation c.258+2t>c under investigation, will be amplified on a heterozygous DNA genomic template and further cloned within the fibronectin EDB intron by exploiting the unique NdeI restriction site. The minigene transcription is driven by the minimal a-globin promoter and SV40 enhancer. Splicing patterns will be evaluated by RT-PCR and sequencing upon lipofection of minigene plasmids in two different cellular models, namely human embryonic kidney cells (HEK293) and hepatocarcinoma cell line (HUH7). Although SBDS is highly expressed in in rapidly proliferating tissues (Zhang 2006), SBDS splicing investigation will be performed without the confounding effect of endogenous SBDS transcripts by using plasmid-specific primers. Splicing patterns obtained with minigenes will be validated by comparison with SBDS splicing patterns evaluated in mRNA from patients' samples. The presence of correct

transcripts will be evaluated in peripheral blood mononuclear cells (PBMC) of homozygous patients. After purification of PBMC, RNA splicing pattern will be evaluated by qPCR and capillary electrophoresis with primers specifically designed to magnify the correct transcripts.

The collection of patients' samples will be favored by the collaboration with Dr. Valentino Bezzerri at Azienda Ospedaliero Universitaria Ospedali Riuniti di Ancona.

These investigations will enable us to verify the presence and the residual levels of correct SBDS transcripts that we hypothesize to be present even in homozygous patients since data in mouse models demonstrated that a SBDS null condition is lethal.

WP1.2_DISSECTION OF SPLICING REGULATORY ELEMENTS:

cis-acting RNA elements are useful targets for deliberate manipulation of pre-mRNA splicing with antisense molecules and, in our case, we will focus on Exonic or Intronic Splicing Silencer (ESS or ISS) or Enhancer (ESE or ISE), sequences able to modulate splicing by recruiting specific splicing factors. The presence and the molecular significance of these splicing regulatory elements will we investigated by using antisense molecules such as Antisense oligonucleotides (AON). AON are single or double stranded molecules, 10-25 nucleotides long, able to base pair to RNA through Watson–Crick base pairing interactions. While they were primarily designed and tested to silence the target gene, they can be efficiently explored to interfere with splicing regulatory elements on pre-mRNA (Wood 2007; Du 2009). We will use 2-O-methyl-phophorothioate oligonucleotides, which have been found to be active in several gene systems. We are aware of the high costs of modified oligonucleotides. For this reason, we will take advantage from the collaboration with Dott.ssa Perrone at the University of Ferrara, who will directly synthesize the oligonucleotide needed for the project.

Evaluation of splicing pattern changes, by RT-PCR, capillary electrophoresis and qPCR, in HEK293 cells transfected with the wild type or mutated minigenes and a panel of different AON spanning all exon and surrounding introns length will allow to identify cis-acting RNA elements regulating the aberrant splicing pattern observed in the presence of the c.258+2t>c nucleotide change.

The dissected splicing regulatory elements will be further validated by site-directed mutagenesis (to destroy the elements) and by silencing of the target protein followed by evaluation of the exon inclusion rate. The collaboration with prof. Perrone, who has a strong experience in AON synthesis, will allow to reduce the overall cost for AON synthesis, an important feasibility element of the project.

WP2: DEVELOPMENT OF RNA-BASED STRATEGIES

We will test a panel of modified U1-snRNA designed to target the mutated 5'ss or downstream intronic sequences (ExSpeU1). Expression vectors for the modified U1-snRNA will be created by mutagenesis

(Balestra 2015).

The identified cryptic splice site, activated by the SBDS c.258+2t>c mutation, or splicing regulatory elements (ISSs) will be targeted with AONs. The ability of U1snRNA and AONs to rescue splicing will be evaluated by co-transfection assays with minigenes followed by qualitative (RT-PCR, capillary electrophoresis, sequencing) and quantitative (real-time RT-PCR) mRNA evaluation (Balestra 2015).

Moreover, combination of modified U1snRNA and AON will be also tested to assess their synergistic effects. When feasible, selected U1-snRNA and AONs will be tested directly in lymphoblastoid cell lines (LCL) from patient upon immortalization or in PHA-activated blasts. As above mentioned, this aspect will take advantage of the collaboration with the Prof. Valentino Bezzeri at University of Verona. Lymphoblastoid cell lines (LCL) are obtained by infection of lymphocytes from HLA-typed donors with culture supernatants of the virus producer B95−8 cell line, cultured in the presence of 0.1 µg/ml of cyclosporin A. Alternatively, PHA-activated blasts are obtained by stimulation of peripheral blood lymphocytes (PBLs) with 1µg/ml of purified PHA for three days and expanded in medium supplemented with IL−2 (Gavioli, 1993). Transfection of these cells with a tracking gene such as Green Fluorescent Protein (GFP) will allow us to evaluate the transfection efficiency. This approach will be conducted in collaboration with Prof. Gavioli at the Department of Life Sciences and Biotechnology of the Ferrara University.

To exclude that the identified ExSpeU1/AON possess a significant toxic effect that might preclude subsequent ex-vivo or in vivo studies, we will exploit classical toxicity assays (MTT), in which cells will be transfected with ExSpeU1/AON and cellular viability evaluated at different time point.

Part E. References

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

Shwachman-Diamond Syndrome (SDS) is a neglected inherited disease affecting 1/168,000 newborns in Italy. Actually, the disease is without a definitive cure, so there is a strong quest for new therapeutic options. The main goal of the project is to investigate the molecular mechanisms associated to SDS caused by the c.258+2t>c mutation and provide preliminary data on rescue of this splicing defect by RNA therapeutics. This will be the first attempt to develop a therapy for SDS patients tailored to specific molecular defect.

UNIVERSITÀ DEGLI STUDI DI FERRARA
Dipartimento di Scienze della Vita e Biotecnologie
Direzione e Segreteria Amministrativa
Via Luigi Borsari, 46
44121 Ferrara

February 28th, 2018

To whom it may concern,

Dr. Dario Balestra has been working at our Institution in the last decade and he is one of the best promising young researchers who has an excellent track of publications in the fields of RNA modulation for therapeutic purposes, a key research topic for our department. Moreover, he is also involved in our teaching activities in the BSc with the course of Recombinant DNA technology, that is highly appreciated by students.

For all these reasons, on behalf of the institution, I strongly encourage his application entitled "*ELUCIDATION OF ABERRANT SBDS SPLICING MECHANISMS TO DESIGN RNA-THERAPEUTICS FOR SHWACHMAN-DIAMOND SYNDROME*", which would boost his independent research and academic career.

Sincerely yours

Prof. Roberto Gambari

Director of the Department of Life Sciences and Biotechnology
University of Ferrara
Via Luigi Borsari, 46
44121, Ferrara, Italy
Phone: +39-0532-974443
email: gam@unife.it

UNIVERSITÀ DEGLI STUDI DI FERRARA
Dipartimento di Scienze della Vita e Biotecnologie
Direzione e Segreteria Amministrativa
Via Luigi Borsari, 46
44121 Ferrara

Dear Sirs,

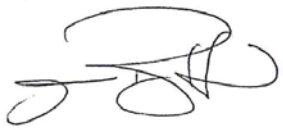
Hereby I'm stating my strong support to the proposal sent by Dr. Dario Balestra and entitled "*ELUCIDATION OF ABERRANT SBDS SPLICING MECHANISMS TO DESIGN RNA-THERAPEUTICS FOR SHWACHMAN-DIAMOND SYNDROME*".

Dr. Balestra has been working in my research group since he entered the PhD program in Biochemistry, Molecular Biology and Biotechnology at the University of Ferrara. During these years he had a pivotal role in the genetic and biochemical characterization of model mutations in the coagulation field as well as other inherited diseases, with the discovery of new aberrant splicing mechanisms and the design of innovative correction strategies acting on pre-mRNA that lay the foundation for the development of RNA-based therapeutic approaches genetic disorders.

He is certainly independent in the design and execution of the experiments and in the analysis and interpretation of results. Among the several PhD students I had the opportunity to mentor, Dr. Balestra has been certainly one of the most talented ones.

I truly believe that this project would represent a great opportunity for his consolidation in the research field and would pave the way for his independent career at our Institution. As an important feasibility element, I declare that he can take advantage of my laboratory space and equipment as well as of the optimized protocols for the in evaluation of human FVIII expression in cellular and animal models.

Sincerely yours
Prof. Mirko Pinotti



Prof. Mirko Pinotti
University of Ferrara
Department of Life Sciences and Biotechnology
Via Fossato di Mortara 74
44121 Ferrara
Italy
Phone.+39 0532 974424
Email pnm@unife.it