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

BIOCHEMICAL AND MOLECULAR INVESTIGATION IN PATIENTS WITH CLINICAL PICTURE OF SDS, BUT WITHOUT *SBDS* MUTATIONS

2**. Applicant Information:**

Name: ANTONELLA MINELLI

Title and Degree(s): PhD

Work Address:
Università degli Studi di Pavia, Dip. di Medicina Molecolare, Unità di Biologia Generale e Genetica Medica, Via Forlanini 14, 27100 PAVIA

Telephone: + 39.0382.987727
Fax: + 39.0382.525030

Email: antonella.minelli@unipv.it

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

Curriculum Vitae

Personal data

Date and place of birth: Roma, July 1st, 1960. Nationality: Italian
Present position: Permanent researcher at Unit of General Biology and Medical Genetics
Institution: University of Pavia, Department of Molecular Medicine , Italy.

Education and training

- January 1984: degree in Biological Science at the University of Pavia, Italy.
- 1984: one-year training post-graduate at the Department of Hereditary and Human Pathology, University of Pavia, to be admitted to State Certification Exam for Biologist, obtained in 1985.
- 1985-1986: fellowship by "Associazione Italiana per lo Studio delle Malformazioni" (ASM) and post-graduate degree training at the Department of Hereditary and Human Pathology.
- May 1987: postgraduate Specialization in Human Cytogenetics, University of Pavia.
- 1988-1990: PhD training performed at the Department of Hereditary and Human Pathology in collaboration with the Department of Biology, University of Milano.
- June 1991: PhD in Human Pathology, University of Pavia.

Teaching experiences

From 1991 she has regularly performed teaching activity (lessons and tutorial activity), in courses of the Medical Faculty including the Schools enabled to provide degrees as Dietologist and Professional Nurse.
From 2001 to 2008 she taught in the Biology course in the Laurea degree in Physical Education.
From 2003 to 2014 she taught in the Biology and Medical Genetics courses in the Laurea degree in Nursing and Obstetrics.
From 2007 to 2010 she taught in the Human Genetics + Biology course in Master's degree of Biomedical Engineering.
From 2011 until now she teaches in the Medical Genetics and Prenatal diagnosis course in the Laurea degree in Biomedical Technician.

Scientific activity

The research activities are mainly focused on the molecular study of myeloproliferative syndromes, including the Shwachman syndrome (SDS) for its tendency in up to one third of cases, to develop myelodysplastic syndrome and acute myeloblastic leukemia; the parental origin of acquired chromosomal anomalies, as observed in haemathological disorders, was also studied.
The current research activity are concerned on the Shwachman syndrome and their topics are:

- mutation analysis of *SBDS* gene;
- study of clonal chromosome abnormalities by genotyping approach in bone marrow and in cells separated from peripheral blood (lymphocytes and granulocytes) to map the origin of chromosomal abnormality in the differentiation of hematopoietic tissue;
- study of cell biology focused on the morphology of neutrophils;
- Whole Exome Sequencing applied in a first group of SDS patients (Ref. 2015-AISS common project submitted by the scientific committee of the AISS).

As collaborator, she obtained funds from

1. Fondazione Cariplo for the project "Analisi genetiche e molecolari per la diagnosi e prevenzione di malattie rare: M. di Rendu-Osler-Weber (ROW) e S. di Shwachman (SS)" Ref.2002.2095/10.8485-08 ATTI 0002- PF RICERCA SCIENTIFICA 2002);
2. Fondazione Cariplo for the project 2012-0529.
3. As principal investigator, she obtained a Research Grant from Associazione Italiana Sindrome di Shwachman (AISS-2011).

4. As principal investigator, she obtained a Research Grant from Associazione Italiana Sindrome di Shwachman (AISS-2016).

From 2003 she provide molecular analysis of *SBDS* gene and her laboratory is present in the Orphanet database (hiip://www.orpha.net).

Published Articles inherent in SDS

1. Valli R, Frattini A, Minelli A. Shwachman-Diamond syndrome: diagnosis, pathogenesis and prognosis. Expert Opinion On Orphan Drugs, 2017; hiips://doi.org/10.1080/21678707.2017.1367282

2. Nacci L, Valli R, Pinto RM, 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 L3MBTL1gene. Genes, Chromosomes & Cancer 2017;56:51–58.

3. 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, Molecules and Diseases.2016;60:33–35.

4. Morini J, Babini G, Mariotti L, Baiocco G, Nacci L, Maccario C, Ro¨ßler U, Minelli A, Savio M, Gomolka M, Kulka U, Ottolenghi A, Danesino C. Radiosensitivity in lymphoblastoid cell lines derived from Shwachman–Diamond syndrome patients. Rad.Prot. Dosimetry. 2015; 166(1–4), 95–100

5. 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;165:573-575.

6. Minelli A, Nicolis E, Cannioto Z, Longoni D, Perobelli S, Pasquali F, Sainati L, Poli F, Cipolli M, Danesino C. Incidence of Shwachman-Diamond syndrome. Pediatr Blood Cancer. 2012;59:1334-1335.

7. Necchi V, Minelli A, Sommi P, Vitali A, Caruso R, Longoni D, Frau MR, Nasi C, De Gregorio F, Zecca M, Ricci V, Danesino C, Solcia E. Ubiquitin-proteasome-rich cytoplasmic structures in neutrophils of patients with Shwachman-Diamond syndrome. Haematologica 2012;97:1057-1063.

8. Maserati E, Pressato B, Valli R, Minelli A, Sainati L, Patitucci F, Marletta C, Mastronuzzi A, Poli F, Lo Curto F, Locatelli F, Danesino C, Pasquali F. The route to development of myelodysplastic syndrome/acute myeloid leukaemia in Shwachman-Diamond syndrome: the role of ageing, karyotype instability, and acquired chromosome anomalies. Br J Haematol. 2009;145:190-197.

9. Minelli A, Maserati E, Nicolis E, Zecca M, Sainati L, Longoni D, Lo Curto F, Menna G, Poli F, De Paoli E, Cipolli M, Locatelli F, Pasquali F, Danesino C. The isochromosome i(7)(q10) carrying c.258+2t>c mutation of the *SBDS* gene does not promote development of myeloid malignancies in patients with Shwachman syndrome. Leukemia. 2009; 23:708-711.

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 01/03/2018

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. Ermanno Gherardi

Title:
Full Professor of General Pathology

Address:
Dipartimento di Medicina Molecolare

Telephone: +390382986845
Fax:

E-mail Address: egherard@unipv.it

_____

Signature of Institution Official 01/03/2018

ABSTRACT OF RESEARCH PLAN

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

TITLE: BIOCHEMICAL AND MOLECULAR INVESTIGATION IN PATIENTS WITH CLINICAL PICTURE OF SDS, BUT WITHOUT *SBDS* MUTATIONS

Objectives and aims.
The project is addressed to obtain an etiological diagnosis in patients lacking mutations in *SBDS* gene but in whom a clinical diagnosis of Shwachman-Diamond Syndrome (SDS) is suggested. This work is also aimed to investigate genetic heterogeneity in SDS.

Background/rationale.
SDS (OMIM#260400) is an autosomal recessive bone marrow failure syndrome. It is characterized by exocrine pancreatic dysfunction, haematologic abnormalities, bone marrow (BM) failure, neurodevelopmental delay and skeletal abnormalities. All the listed clinical problems are observed only in part in the patients, even if they share exactly the same mutations; clinical heterogeneity is observed also among sibs.
Genetic heterogeneity has been recently demonstrated for SDS, with the identification of three new genes, *DNAJC21*, *EFL1* and *SRP54*, in which pathogenic mutations have been found in SDS-like patients.
It is likely that other genes may cause similar phenotypes.
We plan to set up western blot analysis for the identified genes and to performed exome sequencing in a limited number of patients lacking *SBDS* mutations but with clear SDS phenotype.

Research design.
We have already collected, in collaboration with the AISS scientific committee and in collaboration with several Italian groups who are in charge of some SDS patients, clinical data about five cases in whom the clinical picture of SDS is not associated with the presence of *SBDS* mutations.
We already have in our laboratory the expertise to perform western blot analysis, and this method has been applied many times to investigate the presence of the SBDS protein. We will set the method to perform western blot analysis for DNAJC21, EFL1 and SRP54.
In addition we will perform exome sequencing in the previous quoted cases, after obtaining appropriate informed consent and a new blood samples.

Anticipated output.
The results of our project will be: i) to develop western blot methods to be offered to all Italian groups working on SDS, both for diagnostic purposes and research project. In some cases the demonstration that the previously listed proteins are present, will suggest the involvement of other proteins.
ii) in these cases exome analysis is anticipated to identify mutations in new genes, thus expanding the knowledge of genetic heterogeneity in SDS.
Overall, we expect to identify few cases in whom new presently unidentified genes are responsible of the SDS phenotype.

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: Dr. Antonella Minelli Co-I Name: Prof. Cesare Danesino Additional personnel (identify role): A fellowship for 1 year Name to be identify	25,000 euros/year	No request
Equipment: All needed equipment is available	---	---
Supplies: Laboratory supplies (antibodies, consumables, expenses for sequencing)	10.000/year	7.500/year
Other Expenses: General expenses for carrying on the project, bioinformatics expenses	10.000/year	2.500/year
Indirect Costs (not to exceed 10% of total)	1.000/year	
TOTAL COSTS:	46.000/year	10.000/year

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:
The total cost of the project is 46.000 Euros. A salary for a fellowship for 1 year is justified by the amount of laboratory and *in silico* work, which certainly will need full time availability of one person. The following work of validation of the variants and of extension of the analysis of selected variants to a larger set of cases will require 40% of available working time of the PI, 10 % of available working time of the Co-I.

Supplies:
Other: Other expenses include: laboratory consumables (DNA extraction Kit; DNA purification columns; custom sequencing costs; pipet tips, tubes); reagents for DNA amplification and for western blot analysis. General expenses will include all expenses related to the use of dedicated bio-informatic programs, general expenses for running the laboratory, including secretarial expenses and general expenses of the Department, including overheads.

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.

Additional funding needed to complete the project will be received from University of Pavia (salaries to PI and Co-I), and other requests to research dedicate charities are pending.

Research Plan

Part A. Specific aims:

Specific aim of the project will be to obtain an etiological diagnosis in patients lacking mutations in *SBDS* gene, but in whom a clinical diagnosis of Shwachman-Diamond Syndrome (SDS) is suggested, based on international criteria [Dror et al, 2011]. The percentage of cases in whom molecular investigations confirm the clinical hypothesis of SDS is in general quite low. In our experience no more than 20%.

For patients showing a phenotype typical for SDS, about 90 % carry mutation in *SBDS* gene, but about 10 % do not have any mutation in *SBDS* ; thus there is a need for improving the study of genetic heterogeneity in SDS or SDS-like patients.

The aim to identify new genes related to the SDS phenotype can be accomplished by studying both the proteins coded by the known genes and by studying the exome of those patients in whom none of the known genes is mutated. A more general aim of the project will be to improve classification of SDS patients, to obtain better genotype-phenotype correlation, and to understand if severity of the disease may be different in relation to the different genes involved.

Part B. Significance and background :

Shwachman Diamond Syndrome (SDS) is a rare autosomic recessive disorder (OMIM 260400) first described in 1964 by Shwachman and co-workers [1964]. The clinical picture includes exocrine pancreatic insufficiency, hematologic abnormalities, with specific bone marrow chromosomal abnormalities and an increased risk of developing malignant transformation, and skeletal abnormalities.

All clinical signs listed show a wide variability and patients with mild form of the disease and late diagnosis are common. The major disease causing gene is Shwachman-Bodian-Diamond syndrome gene (*SBDS*, OMIM 607444), localized at 7q11.21 [Boocock et al., 2003]. Detection of biallelic pathogenic variants in *SBDS* confirms the clinical diagnosis: three mutations (resulting from gene conversion between *SBDS* and it's pseudogene, *SBDSP*) account for more than 75% of mutated alleles: i) c.183_184TA>CT; ii) c.258+2T>C; iii) c.[183_184TA>CT; 258+2T>C]; in the latter, both DNA changes occur on the same allele. If in a child i) and ii) are found, parents need to be tested to determine whether the mutation(s) observed in their child are monoallelic (as in iii) or biallelic. Targeted mutation analysis is usually performed as this strategy detects at least one mutation in over 90% of patients. About 85% of patients are compound heterozygous for the two common mutations or for a common and a rare mutation; direct sequencing of the five exons of the gene detects less common mutations, including missense, nonsense, insertions, and deletions.

However still about 10% of patient, with clinical features strongly suggesting SDS, remains negative for mutations in *SBDS* [Boocock et al.,2003; Kujipers et al., 2005].

A break into the demonstration of genetic heterogeneity was obtained recently by exome analysis in a small number of cases.

Dhanraj et al. [2017] reported mutations in *DNACJ21* in four patients from Afghani, Canadian and Indian families with an SDS phenotype. Shortly after, Stepensky et al. [2017] reported biallelic mutations in *EFL1* in four cases "SDS like" from three families (Mexican and Palestinian–Muslim families) and Carapito et al. [2017] reported *de novo* dominant mutations in the *SRP54* gene, in three independent Caucasian and Hispanic patients.

SBDS and the new genes identified participate in different steps relevant for the conserved mechanisms of ribosome assembly and protein synthesis.

SBDS protein plays a central role in ribosome biogenesis. It is involved in the maturation of the 60S subunit, coupling GTP hydrolysis by EFL1 on the ribosome, to eukaryotic initiation factor 6 (EIF6) release. This action allows the 80S ribosome assembly and facilitates EIF6 recycling.

SBDS, also, covers many others functions in multiple pathways of mammals. SBDS promotes spindle stability and chromosome segregation as demonstrated by Austin and colleagues [2008]. Depletion of SBDS from human cells sensitizes them to a variety of conditions, including DNA damage and endoplasmic reticulum stress. This finding implies a relevant SBDS function in responses to cellular stress and supports the hypothesis that SBDS is a multifunction protein [Valli et al, 2017].

DNAJC21 belongs to the family of Dna/Hsp40 (heat shock protein 40) proteins, conserved throughout evolution and containing the amino-terminal DnaJ domain through which they bind to Hsp70s. They play an important role in protein translation, translocation, folding, unfolding, and degradation, through the ATPase activity of chaperone proteins Hsp70s [Qiu et al, 2006]. DNAJC21 has been implicated in the late 60S subunit maturation in the cytoplasm [Tummala et al, 2013]. By yeast analysis it has been reported that Jjj1 (ortholog of DNAJC21) removes the shuttling factors Arx1 and Alb1 from the exit tunnel of the pre-60S ribosome subunit. The nascent protein during the translation step must pass through the exit tunnel to exit the ribosome.

EFL1 (Elongation Factor Like GTPase1 [NM_024580.5]) is involved in the biogenesis of the late 60S ribosomal subunit and translational activation of ribosomes. Together with SBDS, EFL1 triggers the GTP-dependent release of EIF6 from 60S pre-ribosomes in the cytoplasm.

Stepensky and co-workers [2017] demonstrated that EFL1 protein of patients with SDS-like symptoms cannot release EIF6 from the 60S surface. The authors hypothesized that the EFL1 mutants cannot transduce GTP hydrolysis into the necessary conformational change to release EIF6 resulting in abnormal ribosomes with altered translating capabilities [Stepensky et al, 2017].

The third gene recently associated to syndromic neutropenia with SDS-like features, *SRP54*, codes for one the major proteins included in the signal recognition particle (SRP). SRP is a ribonucleoprotein (RNP) that in

mammals is composed of a single RNA molecule (the ~300-nucleotide-long 7SL RNA) and the following 6 polypeptides: SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72 [Carapito et al, 2017].

SRP54 plays a central role in this RNP complex binding to SRP19-bound 7SL RNA and associates with the signal peptide of the nascent polypeptide chain. Then, SRP54 binds to the SRP receptor (SR) on the ER membrane and allows the transport of the active ribosome machinery at the surface of the endoplasmic reticulum (ER) [Carapito et al, 2017].

This work, in general, is aimed to investigate genetic heterogeneity in SDS: the number of causal SDS genes reported is very limited if compared to other Inherited Bone Marrow Failure Syndromes (IBMFSs) for which the situation is very different (>20 loci in Fanconi anemia or >10 loci in Diamond-Blackfan anemia) [Carapito et al, 2017].

The diagnostic value of whole-exome sequencing (WES) has been reaffirmed as reported, recently, by Dillon and co-workers [2018]: "as test costs decline, WES has become increasingly used for clinical diagnosis, and now represents the primary alternative to gene panel testing for patients with a suspected genetic disorder". This consideration supports the final part of our project aimed to study those patients, without molecular diagnosis, by exome sequencing.

Part C. Preliminary studies:

Our preliminary results addressed the diagnostic outcome with biochemical and molecular methods. We received in the last four years about 50 requests to perform molecular investigations in patients in whom a diagnosis of SDS was hypothesized. We applied the method of targeted analysis of *SBDS* gene, and identified biallelic mutations in this gene in 7 cases. In four patients only one "common" mutation was identified. In these cases, we performed western blot analysis to verify if the SBDS protein was in fact lacking.

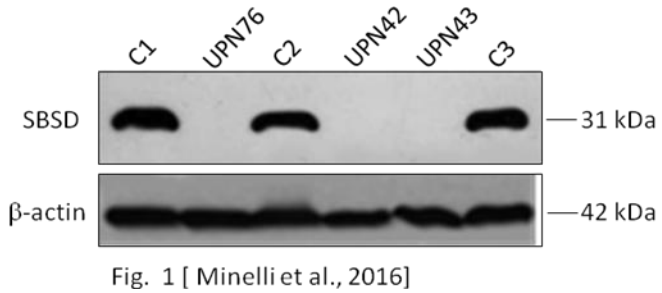

Fig. 1 [Minelli et al., 2016]

Fig. 1. Western blot analysis of SBDS. Example of Western blot in protein lysates from buffy coats of three patients, UPN 42, UPN 43 and UPN 76 and three healthy control samples (C1, C2 and C3). In the bottom β-actin expression as control of good preparation of lysates

As shown in Fig. 1 and 2, we demonstrated that the protein was missing. This result was instrumental to develop new methods of molecular analysis which enabled us to identify in two cases [Minelli et al., 2016], firstly, and later in a third case (Fig.2, Case A) a large deletion (Fig.3) with the same breakpoints. In the forth case, Case B, again SBDS protein is missing (see Fig. 2), but at the time of writing the project, no additional molecular abnormalities were identified.

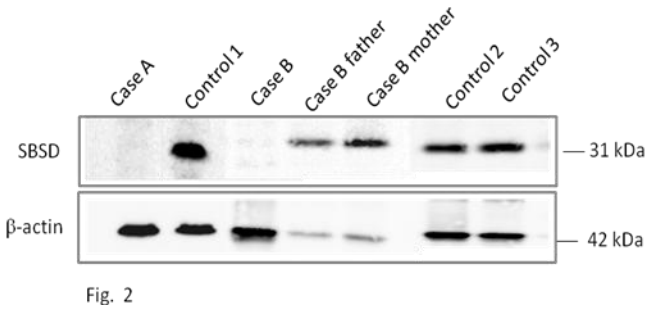

Fig.2. Western blot analysis of SBDS. Example of Western blot in protein lysates from buffy coats of two other patients, Case A and Case B and three healthy control samples (Control 1, Control 2 and Control 3). In the bottom β-actin expression as control of good preparation of lysates.

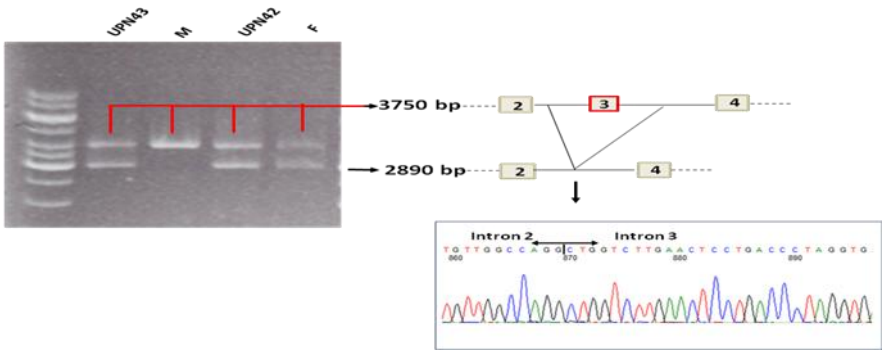

Fig.3

Fig. 3. Example of electrophoretic patterns of amplicons representing exons 2 to 4 obtained by a long-range gDNA PCR. In the figure two sib patients (UPN42 and UPN43) showed two different bands: the first matched a fragment of 3756 bp, containing exons 2, 3 and 4 sequence plus their flanking intronic regions; the second matched a fragment of 2890 bp, corresponding to the deletion of entire exon 3 and part of introns 2 and 3 sequence. F father, M mother

Previous work of our group and from many other colleagues caring for SDS patients, have identified at least five cases in whom a convincing diagnosis of SDS was not followed by the identification of *SBDS* mutations. We also collected a blood sample from a patient in whom a single heterozygous common mutation in the *SBDS* is associated to haematological problems, but in whom the presence of the SBDS protein was demonstrated.

Part D. Experimental design and methods:

The experimental design will include several steps: i) clinical and diagnostic investigations; ii) molecular analyses; iii) biochemical investigations; iv) exome analysis.

i**) clinical and diagnostic investigations**:

the scientific committee of AISS has developed a short form to be filled by colleagues who suspect a diagnosis of SDS in their patients. We use this form to obtain information as to the presence of pancreatic abnormalities, haematological abnormalities, liver function, diabetes, stature skeletal abnormalities, infectious diseases, developmental delay. In patients carrying biallelic mutation in *SBDS*, these information are enough to correlate genotype and phenotype. In cases in whom no *SBDS* mutation s found, we will contact directly the physicians caring for the patient to discuss the phenotype and acquire information on uncommon or unexpected clinical findings, if present. This work is instrumental to be ready to correlate mutations in new genes to clinical variability, but also to discuss additional clinical, laboratory or imaging investigations in patients in whom some clinical data seem not to be related to the SDS or SDS like phenotype.

ii) -a) molecular analysis for the "common" mutations: this molecular analysis will be the first step in all cases. Targeted mutation analysis of exon 2 by PCR-RFLP allows to disclose at one or two of the three common mutations, (c.185_184TA>CT, c.258+2T>C and the combination of [c.183_184TA>CT:258+2T>C] on one allele). Two of the common mutations are observed concomitantly in approximately 62% of SDS patients [Myers, 2014]; the genetic diagnosis will be always completed analyzing the parent of the patient to confirm an independent transmission of mutated alleles;

- b) molecular analyses for uncommon mutations: if only one common mutation has been found, complete sequencing analysis of all five exons of *SBDS* and their flanking intronic regions will be performed as a second step;

- c) molecular analysis for detection of large deletion/duplication: in cases in whom, even after the second step, the second mutation is not found, we will perform as third step a series of long-range PCR on genomic DNA using the same primers of all five exons of *SBDS* but matching them differently to obtain longer amplicons. These amplicons contain more than one exon and can detect the absence or duplication of regions including the annealing sequences of PCR primer used for routine analyses.

If no mutation is found after this additional molecular work, western blot analysis will be performed (see below), and in case of absence of SBDS protein patient-specific additional molecular investigations will be scheduled, as for instance the use of rare cutter restriction endonucleases on large DNA fragments, or FISH investigation with probes specific for the region were the gene is localized.

Details of the methods for mutation analysis are reported in Nicolis et al. [2005] while details for detection of large deletion/duplication are reported in Minelli et al. [2016].

iii) biochemical investigations: thanks to the collaboration with all the colleagues in the scientific committee for Associazione Italiana Syndrome di Shwachman (AISS) and with many Italian clinical centres (headed for instance by Dr. Marco Zecca, IRCCS S.Matteo Pavia; Prof. Franco Locatelli, OPBG Roma; Dr.ssa Vincenzina Lucidi, OPBG Roma; Dr. Carlo Dufour, Gaslini Genova and many others) we will be able to collect a number of blood samples from patients with a convincing clinical picture of SDS but lacking "common" *SBDS* mutations that can be estimated between 3 and 5 cases.

In these cases, in collaboration with the clinicians caring for the patient, we will review the clinical data, to confirm that all diagnostic criteria discussed by Dror et al. [2011] are in fact present and that a different clinical diagnosis has not, in the meantime, been obtained.

On these cases, we will perform western blot analysis for SBDS protein, and we expect to demonstrate that SBDS protein is in fact present; this result of course fits with the absence of *SBDS* mutations.

The second western blot experiment will include the use of antibodies against DNAJC21, EFL1 and SRP54 to check the presence of the coded proteins. All experiments will be done in duplicates.

The demonstration of absence of any of the listed proteins will be followed by sequencing the coding gene.

Methods for western blot analysis: the sample of protein lysates are obtained from buffy coats completely free of red blood cells by repeated washing with ammonium chloride buffer. Immediately after, the lysis of cells is performed and the supernatant are transferred at -20 °C until the use, setting apart 20 ul of it to determine protein concentration using BCA assay with a bovine serum albumen standard curve. For western blots, 40 ug of protein are loaded per lane on a discontinuous SDS-PAGE. The proteins are transferred to a nitrocellulose membrane and then are blocked in 5% milk solution in PBST [Austin et al. 2005]. Antibodies conditions are those recommended by manufacturers.

In cases in whom all the listed proteins are present, we will start, after informed consent, an exome analysis.

iv) exome analysis: in patients in whom a SDS phenotype is present, but without any mutation in the genes up to date known to be related to the development of SDS or SDS-like phenotypes, exome analysis is certainly the "gold standard" type of genetic investigation to identify new genes.

Bamshad et al. [2012] estimated the mean number of coding variants in European Americans: the total number is estimated to be 20,283 (±523) in 100 European Americans, classified as missense (9,511±244), nonsense (93 ± 6), synonymous (10,645 ± 286), splice (34 ± 4). This data allows us to decide the first level of filtering the variants recognized by WES, reducing them to about 50% by excluding all synonymous variants. At this point we will be left with about 100 variants/patient.

The work completed in the wet lab will be followed by estensive *in silico* analysis and we have selected in the KEGG PATHWAY Database the following pathways to be analyzed first:

1) Ribosome (including 137 genes);

2) Ribosome biogenesis in eukaryotes (including 87 genes);
3) Pancreatic secretion (including 96 genes);
4) Development of hematological diseases (82 genes);
5) Stem cell maturation, cell cycle control and regulation of apoptosis (over 100 genes).

Parental DNA will be also collected for all patients cases, so that all variants of interest, after validation with Sanger sequencing, can be studied in "trios" to assess if *de novo* or inherited. When *SRP54* is taken into account, *de novo* variants will have a much higher chance to be causative of the phenotype or to be a strong risk factor as compared to inherited variants.

Methods for exome equencing: we will first quantify the amount of genomic DNA in samples of patients with clinical and biochemical characteristics as previously described; from each patient an informed consent will be obtained. The correct quantification of DNA is essential to proceed with exome analysis: the absorbance measurements at 260 nm are commonly used to quantify DNA, while the ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. DNA quality of each sample will be assessed by gel electrophoresis to exclude the presence of impurities in the sample or damage to DNA. To perform exome analysis an amount of DNA of at least 30 µg of ds DNA in TE, conc. ≥ 300ng/µl is needed. This part of the project, WES analysis, will be performed using custom facilities in collaboration with Prof. E.M. Valente.

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

Our project is relevant for Shwachman Diamond Syndrome because, as first result, we expect to increase our knowledge on its genetic heterogeneity. A second relevant topic is the possibility to identify clinical differences related to mutations in different genes. These differences will be of utmost importance if they will concern the prognosis or risk to develop haematological malignancies. Of course, a third relevant outcome will the possibility to offer to a larger number of families precise genetic counselling, and, if requested, prenatal diagnosis.

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

Austin KM, Leary R.J, Shimamura A. The Shwachman–Diamond SBDS protein localizes to the nucleolus. Blood. 2005; 106: 1253–1258.

Austin KM, Gupta ML Jr, Coats SA, et al. Mitotic spindle destabilization and genomic instability in Shwachman-Diamond syndrome. J Clin Invest. 2008;118(4):1511–1518.

Bamshad MJ, Ng SB, Bigham AW, Tabor HK, Emond MJ, Nickerson DA, Shendure J. Exome sequencing as a tool for Mendelian disease gene discovery.Nat Rev Genet. 2011;12(11):745-755.

Boocock GR, Morrison JA, Popovic M, Richards N, Ellis L, Durie PR & Rommens JM. Mutations in SBDS are associated with Shwachman-Diamond syndrome. Nature Genetics. 2003; 33:97–101.

Carapito R, Konantz M, Paillard C, Miao Z, Pichot A, Leduc MS, et al. Mutations in signal recognition particle SRP54 cause syndromic neutropenia with Shwachman-Diamond-like features. J Clin Invest. 2017 Nov 1;127(11):4090-4103.

Dhanraj S, Matveev A, Li H, et al. Biallelic mutations in DNAJC21 cause Shwachman-Diamond syndrome. Blood. 2017;129(11):1557–1562.

1: Dillon OJ, Lunke S, Stark Z, Yeung A, Thorne N; Melbourne Genomics Health Alliance, Gaff C, White SM, Tan TY. Exome sequencing has higher diagnostic yield compared to simulated disease-specific panels in children with suspected monogenic disorders. Eur J Hum Genet. 2018 doi: 10.1038/s41431-018-0099-1..

Dror Y, Donadieu J, Koglmeier J, Dodge J, Toiviainen-Salo S, Makitie O, Kerr E, Zeidler C, Shimamura A, et al. Draft consensus guidelines for diagnosis and treatment of Shwachman-Diamond syndrome. Ann N Y Acad Sci. 2011; 1242:40-55.

Kuijpers TW, Alders M, Tool AT, Mellink C, Roos D, Hennekam RC. Hematologic abnormalities in Shwachman Diamond syndrome: lack of genotype-phenotype relationship. Blood. 2005 Jul 1;106(1):356-61.

Minelli A, Nacci L, Valli R, et al. Structural variation in SBDS gene, with loss of exon 3, in two Shwachman-Diamond patients. Blood Cells Mol Dis. 2016;60:33–35.

Myers K., Shwachman-Diamond syndrome, in: R.A. Pagon, M.P. Adam, H.H. Ardinger,et al., (Eds.), GeneReviews® [Internet], University of Washington, Seattle, (1993–2016), Seattle (WA), 2008 (Updated 2014 Sep 11).

Nicolis E, Bonizzato A, Assael BM, Cipolli M. Identification of novel mutations in patients with Shwachman-Diamond syndrome. Hum Mutat. 2005;25(4):410.

Qiu XB1, Shao YM, Miao S, et al. The diversity of the DnaJ/Hsp40b family, the crucial partners for Hsp70 chaperones. Cell Mol Life Sci. 2006;63:2560–2570.

Stepensky P, Chacón-Flores M, Kim KH, et al. Mutations in EFL1, an SBDS partner, are associated with infantile pancytopenia, exocrine pancreatic insufficiency and skeletal anomalies in a Shwachman-Diamond like syndrome. J Med Genet Published Online First. 2017; March 22. Doi: 10.1136/jmedgenet-

2016-104366.

Shwachman H, Diamond LK, Oski FA, et al. The syndrome of pancreatic insufficiency and bone marrow dysfunction. J Pediatr.1964;65:645–663.

Tummala H, Walne AJ, Williams M, et al. DNAJC21 mutations link a cancer-prone bone marrow failure syndrome to corruption in 60S ribosome subunit maturation. Am J Hum Genet. 2016;99:115–124.

Valli R, Frattini A, Minelli A. Shwachman-Diamond syndrome: diagnosis, pathogenesis and prognosis. Expert Opinion On Orphan Drugs, 2017; hiips://doi.org/10.1080/21678707.2017.1367282