Associazione Italiana Sindrome di Shwachman-Diamond (AISS)
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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: ROLE OF NOTCH PATHWAY IN SHWACHMAN-DIAMOND SYNDROME HAEMATOPOIESIS

2**. Applicant Information:**

Name:
Simone Cesaro, M.D.

Title and Degree(s):
Medical doctor, specialist in Pediatrics, Director of Pediatric Hematology Oncology Unit, Azienda Ospedaliera Universitaria Integrata

Work Address:
Piazzale L.A.Scuro 10, 37134, Verona

Telephone: 045-8124931-6889

FAX: 045-8126326

Email: simone.cesaro@ospedaleuniverona.it

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

Cesaro Simone, short CVC
Simone Cesaro, M. D.
Born: 12 January 1962
Place of birth: Padua
Nationality and citizenship: Italian

PRE-MEDICAL EDUCATION

Scientifically oriented High School at Liceo B.B. Ferrari in Este (Padova), 1976-81

MEDICAL EDUCATION

-School of Medicine of University of Padua, 1981-1987.
-Fellow student at 2nd Clinic of Pediatrics, 1985-1988
-Qualified with honours as M. D. at the Medical School of University of Padua (Italy), March 30th 1988.
-Military Service as Medical Officer in Italian Army, 1988-1989 (15 months)
-Resident at the School of Paediatrics, Department of Paediatrics, University of Padua, 1988-1992
-Specialization in Paediatrics, School of Paediatrics, University of Padua, December 11th , 1992
-Fellow at 2nd Clinic of Pediatrics, Division of Hematology Oncology, 1990-1994
-Medical Assistant at Pediatric Hematology-Oncology Division, University of Padua, from February 1994 to October 2009

PRESENT POSITION

-Director of Pediatric Hematology Oncology, Policlinico GB. Rossi, Azienda Ospedaliera Univeristaria Integrata, Verona

SCIENTIFIC AFFILIATIONS

-Italian Association of Paediatric Hematology-Oncology (AIEOP)
-Italian Association of Pediatrics (SIP)
-International Immunocompromised Host Society (ICHS)
-Multinational Association of Supportive Care in Cancer (MASCC)
-European Group for Blood and Marrow Transplantation (EBMT)
-American Society for Blood and Marrow Transplantation (ASBMT)

AREAS OF INTEREST

-Supportive care in cancer patients, in particular the prophylaxis and treatment of infectious diseases and management of complications of central venous catheter
-Hematopoietic stem cell Transplantation

PUBLICATIONS

see Google Scholar public prophile at:
https://scholar.google.it/citations?user=iSsewwsAAAAJ&hl=it

CURRENT APPOINTMENTS

-Chairman of Infectious Disease Working Group of Italian Association of Pediatric Hematology Oncology (AIEOP)
-Member of Supportive Care Committee of Italian Association of Pediatric Hematology Oncology (AIEOP)
-Member of Infectious Disease Working Party of European Group for Blood and Marrow Transplant (EBMT)
-Member of Aplastic Anemia Working Party of European Group for Blood and Marrow Transplant (EBMT)
-Member of Pediatric Diseases Working Party of European Group for Blood and Marrow Transplant (EBMT)
-Medical Advisor of Lifeline Italia O.N.L.U.S.

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I authorize to use my personal data according to the D.L. 196/2003

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.

The project will be submitted to Ethics Committee after approval of the project and of budget plan by AISS

6**. Research Results**:

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

Simone Cesaro

_____ 21/12/2016

Signature of Applicant 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: Pediatric Hematology Oncology, Azienda Ospedaliera Universitaria Integrata Verona

Title: M.D. Director of Unit

Address: Piazzale L.A. Scuro 10, 37134, Verona

Phone: 045-8124931-6889 Fax: 045-812.6326

E-mail Address: simone.cesaro@ospedaleuniverona.it

Simone Cesaro

_____ 21/12/2016

Signature of Institution Official Date

ABSTRACT OF RESEARCH PLAN

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

TITLE: ROLE OF NOTCH PATHWAY IN SHWACHMAN-DIAMOND SYNDROME HAEMATOPOIESIS

Scientific Aims:

1. To assess the ability of CD34+ cells obtained from bone marrow of Shwachman-Diamond Syndrome (SDS) patients to form colonies in a semi-solid medium;
2. To evaluate the contribution of Notch signalling to the defective haematopoiesis observed in SDS patients;
3. To perform RNA sequencing (RNA-seq) on CD34+ cells from SDS patients in order to screen deregulated genes that may explain defective haematopoiesis associated to SDS.

Methodology of the Proposal:

The project consist of three phases:

1. Isolation of CD34+ cells from BM of SDS patients to assess their different ability to form colonies in a semi-solid medium compared to CTRL CD34+.

2. Treatment of SDS CD34+ cells with modulators of Notch signalling to understand and evaluate the real involvement of Notch signalling in abnormal SDS haematopoiesis. The treated cells will be used to perform CFU assay to evaluate any potential change in their clonogenic abilities. Normal BM will be used as a control.

3. RNA-seq of SDS and CTRL CD34+ cells to evaluate differentially expressed genes involved in haematopoiesis.

The duration of the study is 2 years. We plan to work on 20 samples over these two years.

Long-term Objectives:

The demonstration of possible role of Notch signalling in SDS haematopoiesis paves the way to the use of Notch modulators as futures drugs for the treatment of SDS patients. The establishment of a gene pattern expression of SDS CD34+ may highlight and propose new candidates genes that could be associated with the abnormal SDS haematopoiesis.

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: Simone Cesaro (MD)	Free involvement	Free involvement
Co-I Name: Mauro Krampera(MD,PhD)	Free involvement	Free involvement
Additional personnel (identify role):		
Name: Angela Mercuri (Research Fellow)	4000€	1000€/year
Name: Annalisa Adamo (PhD student)	Free involvement	Free involvement
Name: Roberta Carusone (PhD, Post Doctoral Fellow)	Free involvement	Free involvement
Name: Paul Takam Kamga (PhD, Post Doctoral Fellow)	Free involvement	Free involvement
Equipment:		
Supplies:	24000€	8500€/ year

Other Expenses:		
Indirect Costs (not to exceed 10% of total)	1000 €	500 €/year
TOTAL COSTS*:	29000€	10000€/year

* The duration of the study is 2 years. The total cost required to complete the project is 29000€. We are currently asking a funding for the first year (10000€). So we plan to ask a new funding for the second year, of course by presenting the results obtained the first 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.

Supplies Include:

- Modulators of Notch signaling;
- Medium for CFU-assay;
- CD34+ isolation kit;
- RNA isolation and Real Time qPCR Kits;
- RNA-sequencing and data analyses;
- Plastics.

Personnel:

- Angela Mercuri will perform the laboratory work in this study

PERSONNEL	ROLE IN THE PROJECT
Simone Cesaro	Principal Investigator and responsible for the research
Mauro Krampera	Co-principal investigator Supervisor of the study on the functional role Notch signalling in haematopoiesis
Angela Mercuri	Planning and execution of experiments under the guidance of Investigators
Annalisa Adamo	Involved in transcriptomic studies
Roberta Carusone	Involved in in vitro studies of haematopoiesis
Paul Takam Kamga	Involved in experimental pharmacology

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.

Grant from Fondazione Cariverona 2012

Research Plane

PART A. SPECIFIC AIMS

1. To assess the ability of CD34+ cells obtained from bone marrow of Shwachman- Diamond Syndrome (SDS) patients to form colonies in a semi-solid medium;

2. To evaluate the contribution of Notch signalling to the defective haematopoiesis observed in SDS patients;

3. To perform RNA sequencing (RNA-seq) on CD34+ cells from SDS patients in order to screen deregulated genes that may explain defective haematopoiesis associated to SDS.

PART B. SIGNIFICANCE AND BACKGROUND

Shwachman-Diamond Syndrome (SDS) is a rare autosomal recessive disorder characterized by exocrine pancreatic insufficiency, skeletal abnormalities and bone marrow (BM) failure, which is associated to various degrees of peripheral cytopenias (1, 2). In addition, SDS patients have an increased risk to develop Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML) (3). BM from SDS patients shows abnormalities both in the number and in the function of stem/progenitors cells (HSCs/HPCs), suggesting a defective haematopoiesis (4). Indeed, BM from SDS patients is characterized by decreased frequency of CD34+ cells which have reduced capacity *in vitro* to form hematopoietic colonies of all lineages (*5)*. Developmental pathways, such as Notch signalling, support normal haematopoiesis and are critical for the development of haematological diseases (6,7). Notch family consists of four Notch receptors (Notch 1, Notch 2, Notch 3, Notch 4) and five Notch ligands (Jagged 1, Jagged 2, Delta-like 1, Delta-like 3, Delta-like 4). The canonical NOTCH pathway is initiated by the binding of ligand to Notch receptor expressed in the neighbouring cells. This interaction induces the proteolytic cleavage of Notch protein, resulting in the release of its intracellular domain; the latter translocates to the nucleus where it initiates transcription of target genes controlling hematopoietic stem cell self-renewal, proliferation and differentiation (8). Abnormal functioning of Notch pathway is associated to defective haematopoiesis, as observed in many haematological diseases including T-ALL, CLL, and AML (7). We hypothesize that poor haematopoiesis in SDS patients could be supported by aberrance in Notch pathway; as a consequence, the pharmacological modulation of Notch could improve the haematopoietic defects in SDS patients.

PART C. PRELIMINARY STUDIES

Haematopoiesis is a phenomenon that depends on a dynamic interaction between HSCs and BM microenvironment components, such as Mesenchymal Stromal Cells (MSCs) (9). Growing evidence clearly demonstrated that during haematopoiesis Notch signalling mediates this interaction (10). In a preliminary study using flow cytometry, we evaluated the expression of Notch receptors and their ligands on CD34+ cells as well as on MSCs from SDS patients, suggesting a possible involvement of Notch signalling in physiopathology of the disease. We then decided to set up functional studies in order to clarify the real involvement of Notch signalling in SDS haematopoiesis. Colony Forming Cells (CFU) assay is a functional study used to evaluate the differentiation pattern of hematopoietic progenitors to form colony in a semisolid medium (11). This assay is a gold standard for the *in vitro* detection and quantification of

hematopoietic progenitor cells. Our preliminary observation with BM samples from two SDS patients showed that SDS CD34+ cells were unable to form colonies in CFU assay as compared to controls (CTRLs). These observations should be confirmed on a larger number of SDS patients in presence of pharmacological modulators of NOTCH signalling in the semisolid medium. Considering that Notch signalling is not the only pathway involved in the regulation of haematopoiesis, we would like also to perform the RNA-sequencing (RNA-seq) on CD34+ cells from SDS patients, in order to screen deregulated genes that may explain the defective haematopoiesis associated to SDS.

PART D. EXPERIMENTAL DESIGN AND METHODS

The project consist of three phases:

1. Isolation of CD34+ cells from BM of SDS patients to assess their different ability to form colonies in a semi-solid medium compared to CTRL CD34+.
2. Treatment of SDS CD34+ cells with modulators of Notch signalling to understand and evaluate the real involvement of Notch signalling in abnormal SDS haematopoiesis. The treated cells will be used to perform CFU assay to evaluate any potential change in their clonogenic abilities. Normal BM will be used as a control.
3. RNA-seq of SDS and CTRL CD34+ cells to evaluate differentially expressed genes involved in haematopoiesis.

The duration of the study is 2 years.

Materials and methods Patients

and cells

After informed consent, patients affected by SDS will undergo BM aspirate in our Unit or in any other site that will be involved in the study. Control samples will be obtained from healthy donors or patients with solid tumors, but without any haematological diseases. CTRL BM will be age-matched with BM from SDS patients. We plan to work on 20 samples over 2 years.

Isolation of CD34+ cells: Mononuclear cells will be isolated from human BM using Ficoll. Purified CD34+ will be isolated from mononuclear cells using appropriate positive selection kit. The purity and vitality of CD34+ cells will be evaluated by flow cytometry.

CFU assay: Purified CD34+ cells will be plated in a semi-solid methylcellulose medium, which is standardized to promote optimal growth and differentiation of hematopoietic progenitor cells over a culture period of at least 14 days. Then the colonies will be classified and enumerated *in situ* by light microscopy. The number and the morphology of the colonies formed by a fixed number of cells will provide information about the ability of progenitors to differentiate.

Treatment of CD34+ cells: To evaluate the role of Notch signalling on the clonogenic capacity of SDS CD34+, purified CD34+ will be treated for 24 hours with Notch modulators or their specific vehicles and then plated in a semi-solid methylcellulose medium for CFU assay.

CD34+ RNA isolation and sequencing: Total RNA will be isolated from CD34+ cells using an appropriate isolation kit. RNA-Seq will be performed at Functional Genomics Laboratory of the University of Verona after enrichment of

poly(A) RNA using the TruSeq kit (Illumina). After conversion to double stranded cDNA using random hexamer-primers, sequencing adaptors will be ligated to the fragments and the generated libraries will be subsequently enriched by PCR amplification. The library products will be sequenced on an Illumina NextSeq500 sequencer using 75bp single-end reads, generating 20 million clean reads per sample. After quality filtering, sequencing reads will be aligned to the reference human genome and differential expression analysis will be performed using DESeq2.

In vitro validation of candidate mRNAs: Once genes differentially expressed are identified, they will be firstly evaluated *in silico* using different pathway prediction tools. Selected mRNAs will be validated by real time qPCR.

The RNA-seq will be performed only of 5 SDS patients versus 5 controls. However, the validation by qPCR of selected differentially expressed genes will be done on the 20 patients involved in this study.

PART E. REFERENCES

1. Shwachman H, Diamond LK, Oski FA, Khaw KT. The syndrome of pancreatic insufficiency and bone marrow dysfunction. J Pediatr 1964; 65: 645-663.
2. Mäkitie O, Ellis L, Durie PR, Morrison JA, Sochett EB, Rommens JM, Cole WG. Skeletal phenotype in patients with Shwachman-Diamond syndrome and mutations in SBDS. Clin Genet 2004; 65:101-112.
3. Smith OP, Hanna IM, Chessels JM, Reeves BR, Milla P. Haematological abnormalities in Shwachman-Diamond syndrome. Br J Haematol 1996; 94:279-284.
4. Mercuri A, Cannata E, Perbellini O, Cugno C, Balter R, Zaccaron A, Tridello G, Pizzolo G, De Bortoli M, Krampera M, Cipolli M, Cesaro S. Immunophenotypic analysis of hematopoiesis in patients suffering from Shwachman-Bodian-Diamond Syndrome. Eur J Haematol 2015; 95:308-315.
5. Dror Y, Freedman MH. Shwachman-Diamond syndrome: An inherited pre- leukemic bone marrow failure disorder with aberrant hematopoietic progenitors and faulty microenvironment. Blood 1999; 94:3048–3054
6. Milner LA, Bigas A. Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. Blood 1999; 93:2431-2448.
7. Ntziachristos P, Lim JS, Sage J, Aifantis I. From fly wings to targeted cancer therapies: a centennial for notch signaling. Cancer Cell 2014; 25:318-334.
8. Suresh S, Irvine AE. The NOTCH signaling pathway in normal and malignant blood cell production. J Cell Commun Signal 2015; 9:5–13.
9. Shiozawa Y, Havens AM, Pienta KJ, Taichman RS. The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. Leukemia 2008; 22:941-950.
10. Saleh M, Shamsasanjan K, Movassaghpourakbari A, Akbarzadehlaleh P, Molaeipour Z. The Impact of Mesenchymal Stem Cells on Differentiation of Hematopoietic Stem Cells. Adv Pharm Bull. 2015; 5: 299–304.
11. Sarma NJ, Takeda A, Yaseen NR. Colony forming cell (CFC) assay for human hematopoietic cells. J Vis Exp 2010; (46): 2195.

12. Ginzberg H, Shin J, Ellis L, et al. Shwachman syndrome: phenotypic manifestations of sibling sets and isolated cases in a large patient cohort are similar. J Pediatr. 1999; 135:81-88

PART F. RELEVANCE OF THE RESEARCH TO SHWACHMAN-DIAMOND SYNDROME

Patients with SDS suffer from haematological abnormalities including neutropenia, anemia, thrombocytopenia, or trilineage cytopenia (12). In addition, SDS patients have increased risk to develop MDS and AML (3). Due to the complexity of clinical manifestation, the rarity and the heterogeneity of BM progenitors, the affected cell types and altered genetic networks remain unknown. The demonstration of possible role of Notch signalling in SDS haematopoiesis paves the way to the use of Notch modulators as futures drugs for the treatment of SDS patients. The establishment of a gene pattern expression of SDS CD34+ may highlight and propose new candidates genes that could be associated with the abnormal SDS haematopoiesis.

PART K.

The project will be submitted to Ethics Committee after approval of the project and of budget plan by AISS