Highlights from EHA 2015
Firenze 18-19 Settembre

Globuli Rossi
MD Cappellini
A. Iolascon
Disclosures

Member of advisory board for Novartis, Celgene, Sanofi/Genzyme
Global epidemiology of hemoglobinopathies: New management challenges  
Speaker: F Piel

Myths and Facts on the Management of Iron Overload  
Speaker: A Kattamis

Cure for thalassemia major – From allogeneic hematopoietic stem cell transplantation to gene therapy  
Speaker: A Srivastava
Red Cells EHA 2015

Heme trafficking in iron metabolism: Notes from the underground
Speaker: I Hamza

From Disease Models Of Abnormal Iron Metabolism And Erythropoiesis To Novel Therapies
Speaker: S Rivella

Heme and erythropoiesis
Speaker: D Chiabrando
Iron a global issue in Hematology
C. Camaschella

New Generation Sequencing In anemias
R Van Wiyck

Iron overload in rare anemias
M Muchenthaler

New perspective in treatment of Hbpathis
MD Cappellini
Focus on

- New Perspectives for hemoglobinopathies treatment including gene therapy
- Interim results with Sotatercept
- New generation sequencing in anemias
New approaches to Thalassaemia treatment

• Gene Therapy
• Gene Therapy based on HbF induction
• Regulation of erythropoiesis
Gene therapy: objective

Gene transfer of β-globin in stem cells to reduce the α–β imbalance in erythroid cells

Increase of normal RBCs and reduction of ineffective erythropoiesis

Transfusion independence

β-globin gene

Red blood cells
Source of stem cells

—mobilization is the preferred source of stem cells for gene therapy of thalassaemia

• G-CSF: safe and efficient mobilization of non-splenectomized patients

• plerixafor: safe and efficient mobilization of splenectomized patients

# Ongoing and planned thalassaemia gene therapy trials

<table>
<thead>
<tr>
<th>Trial</th>
<th>Patients</th>
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<tr>
<td>Paris</td>
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<tr>
<td>Cincinnati</td>
<td>Adults and children</td>
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<tr>
<td>Memphis</td>
<td>Adults</td>
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<tr>
<td>New York</td>
<td>Adults</td>
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<td>Adults and children</td>
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<td>Thessaloniki</td>
<td>Adults</td>
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Northstar (HGB-204) Study

• Non-randomized, open-label, international, multi-center, Phase 1/2 study in adults with β-thalassemia major
  – Age 18-35 years at the time of consent
  – Transfused with ≥ 100 mL/kg/year of pRBCs or transfused ≥ 8 times in each of the preceding 2 years
  – Sites: U.S. (4), Australia (1), Thailand (1)

• Centralized stem cell transduction with LentiGlobin BB305 lentiviral vector

• Primary objectives: Safety and efficacy of LentiGlobin BB305 Drug Product for the treatment of β-thalassemia major
  – Primary endpoint: ≥ 2g/dL of Hb $A^{T87Q}$ at 18-24 months post-infusion
  – Safety endpoints: Clinical and laboratory adverse events, Replication competent lentivirus (RCL), insertion site analysis (ISA), oncogenesis
LentiGlobin BB305 Lentiviral Vector

- Replication defective, self-inactivating lentiviral vector that produces β\(^{A-T87Q}\)-globin using erythroid-specific globin gene regulatory elements

- An amino acid substitution (β\(^{A-T87Q}\)) allows for HPLC monitoring of transgene globin levels in the patient’s cells
Summary Gene therapy: where are we now?

- **Advantages**
  - no need for matched donor
  - eliminates risks of GVHD and graft rejection

- **Challenges**
  - optimal methods for bone-marrow conditioning
  - safe and efficient gene transfer and engraftment
  - lack of selection advantage for corrected cells
  - consistent, safe, and therapeutic haemoglobin production in lineage-specific manner

- **Achievements**
  - correction of mouse haemoglobinopathies using ex vivo transduction of HSC with β-globin lentiviral vectors
  - 12 patients with severe β-thalassaemia treated using lentiviral β-globin gene transfer

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GVHD, graft-versus-host disease.

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Domanda 1

• Quale è il vostro feeling nei confronti della terapia genica:
  - rispetto al trapianto di midollo
  - costo/beneficio
New approaches to Thalassaemia treatment

- Gene Therapy
- Gene Therapy based on HbF induction
- Regulation of erythropoiesis
- Iron chelation
Introduction of GG1-Ldb1 into adult primary human erythroid cells strongly activated γ-globin expression with a concomitant reduction in β-globin transcription. Strikingly, γ-globin accounted for nearly 90% of total β-type globin transcription.
Recruitment of LCR by Ldb1 fused to a ZF that binds specific sequences on γ-gene

Redirecting the developmental gene expression switch in favor to gamma globins has therapeutic implications

Vettore pCL20cAnkyrinGG1DDiGFP

Rivellea S. et al EHA 2015
Shift of globin mRNA expression in vitro (cultures of Cd34 from SCD patients)

Shift dell’ espressione dell’mRNA delle globine

HbS $\rightarrow$ HbF switch
These results demonstrate the power of forced chromatin looping to reprogram developmental regulation of gene expression.

It provide a novel proof of concept for activating the γ-globin gene for the benefit of patients with hemoglobinopathies.
New approaches to Thalassaemia treatment

- Gene Therapy
- Gene Therapy based on HbF induction
- Regulation of erythropoiesis
Sotateceupt (ACE-011)
Luspatercept (ACE-536)
Sotatercept (ACE-011)

- Recombinant human fusion protein consisting of the ECD of ActRIIA linked to the human IgG1 Fc domain

- Competes with the activin receptor IIA and binds a number of TGF-β superfamily ligands including activins, BMPs and GDFs

- A murine surrogate molecule referred to as RAP-011 was costructed by exchanging the human IgG1 Fc sequence portion of sotatercept with its murine IgG2a homolog

ECD, extracellular domain; ActRIIA, activin receptor type IIA; EPO, erythropoietin; GDF, growth differentiation factor; IgG, immunoglobulin G; TGF-β, transforming growth factor beta.
ACE-536

- ACE-011, ACE-536 promote late-stage erythroid differentiation via a mechanism distinct from ESAs (Suragani R et al., Nature Med 2014)

**Sotatercept**
- ECD of ActRIIA
- Fc Domain of human IgG\(_1\) Antibody

**ACE-536**
- Modified ECD of ActRIIB
- Fc Domain of human IgG\(_1\) Antibody

- ACE-536 and sotatercept bind to various ligands in the TGF-β superfamily with differing affinities
  - Both bind to GDF11 and inhibit Smad 2,3 signaling

ESA, erythroid stimulating agent; ECD, extracellular domain; TGF-β, transforming growth factor β; GDF, growth differentiation factor
Key Findings From Prior Preclinical and Clinical Studies

- Sotatercept was initially evaluated as an anabolic bone agent\textsuperscript{1}

- Pharmacodynamic effects include increased bone mass (anabolic and anti-resorptive\textsuperscript{2}) and increased red cell parameters, as reported in multiple animal models and a phase I study of healthy postmenopausal women\textsuperscript{3}

AEs=adverse events.

Key Findings From Prior Preclinical and Clinical Studies

• Administration of ACE-011 to monkeys or mice has resulted in the reversal of bone loss and osteoporosis (Lotinun et al, Bone. 2010)

• In healthy postmenopausal women, sotatercept therapy was associated with increased RBC parameters, including Hb level (Sherman ML, et al. J Clin Pharmacol. 2013)

  
  Model: Transgenic β-thalassemia intermedia mouse model (Hbb\textsuperscript{th1/th1})
  
  Treatment: RAP-011 10mg/kg twice/week for 60 days
  
  Outcome: Corrected anemia
            Improved ineffective erythropoiesis
            Decreased spleen weight and cellularity
            Improved RBC morphology
            Decreased iron overload
Interim Results From a Phase 2a, Open-Label, Dose-Finding Study to Determine the Safety, Efficacy, and Tolerability of Sotatercept (ACE-011) in Adults With β-Thalassemia

Objectives

To determine a safe, tolerable, and effective dose of sotatercept in adult patients with β-thalassemia major who are transfusion dependent (TD), and adult patients with β-thalassemia intermedia who are TD or non-TD (NTD
Results: NTD Patients

(S137) Interim results from phase 2A, open-label, dose-findings study od Sotatercept (Ace 011) in adult patients with Beta Thalassemia

MD Cappellini
Results: Dose-dependent Hb Increase in NTD Thalassemia Patients

Number of patients with sotatercept dose (mg/kg)

<table>
<thead>
<tr>
<th>Sotatercept dose</th>
<th>1 year</th>
<th>2 years</th>
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<td>0.1 mg/kg</td>
<td>6 6 6 5 5 3 3 3 3 3 3 3 3 3 2 2 2 1</td>
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<tr>
<td>0.3 mg/kg</td>
<td>6 6 6 6 6 6 6 6 6 6 6 6 6 5 5 5 4 4 3 2 2 2 2 2 2 2 2 2 2 2 2 1</td>
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<tr>
<td>0.5 mg/kg</td>
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<td>0.75 mg/kg</td>
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<td>1.0 mg/kg</td>
<td>5 5 5 5 4 4 3 2 2 2 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1</td>
<td></td>
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Interim data as of May 6, 2015.
Results: NTD Thalassemia Patients With Hb Increase Sustained for ≥ 12 Weeks

Interim data as of May 6, 2015.

<table>
<thead>
<tr>
<th>Sotatercept dose</th>
<th>≥ 1.0 g/dL Hb increase</th>
<th>≥ 1.5 g/dL Hb increase</th>
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<tbody>
<tr>
<td>0.1 mg/kg (n = 6)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>0.3 mg/kg (n = 6)</td>
<td>67%</td>
<td>33%</td>
</tr>
<tr>
<td>0.5 mg/kg (n = 6)</td>
<td>67%</td>
<td>33%</td>
</tr>
<tr>
<td>0.75 mg/kg (n = 7)</td>
<td>86%</td>
<td>71%</td>
</tr>
<tr>
<td>1.0 mg/kg (n = 5)</td>
<td>20%</td>
<td>20%</td>
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</table>

(n = number of patients in each dose group)
Results: RBC Morphology Before and After Treatment

- RBC morphology before treatment (A), after 6 months treatment (B), and after 30 months treatment (C) with sotatercept 0.5 mg/kg in a splenectomized 40-year-old Italian male
  - At baseline, Hb was 9.2 g/dL, MCH 29.0 pg, MCV 83.9 fl, RDW 23.3%
  - At 2 months, Hb was 10.2 g/dL, MCH 28.7 pg, MCV 83.1 fl, RDW 24.5%
  - At 19 months, Hb was 10.7 g/dL, MCH 28.1 pg, MCV 86.4 fl, RDW 22.8%

MCH, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red blood cell distribution width.
Patient 0203 – Leg Ulcer

ACE-536 β-Thalassemia Phase 2 Clinical Trial

- 30 year old male, non-transfusion dependent thalassemia intermedia
- Baseline Hb 9.2 g/dL
- History of lower limb ulcers since 2011
  - Leg ulcer healing noticed 2 weeks after first dose of ACE-536 (0.4 mg/kg)
  - 2nd dose delayed due to unrelated bone marrow hypoplasia
  - Leg ulcer substantially resolved after 6 weeks on treatment
  - Patient received a total of 4 doses; maximum Hb on study 10.6 g/dL

Pre-Treatment

After 6 Weeks ACE-536

Study Day

A.Piga EHA 2014
Results:
TD Patients

(S136) Luspatercept (Ace 356) increases hemoglobin and decreases transfusion burden and liver iron concentration in adult with Beta-thalassemia: preliminary results from phase 2 study

A. Piga
Results: Reduction in Transfusion Burden for TD β-Thalassemia Patients

- Mean transfusion burden reduction among patients treated with sotatercept ≥ 0.5 mg/kg was 32.25%

*a Percentage change in transfusion burden (units/168 days) from baseline to on-treatment.

Interim data as of May 6, 2015.
Results: Erythroid Response Correlates With Serum Sotatercept Exposure

- No apparent effects of weight, sex, age, or transfusion burden on drug clearance were observed

Interim data as of April 1, 2015.
Where Does Sotatercept Impact Erythropoiesis?

How much EPO is required?
Conclusions

- These data suggest that long-term treatment with sotatercept can:
  - Increase Hb levels in NTD patients
  - Reduce transfusion burden in TD patients
- Increase in Hb level and reduction in transfusion burden correlated with increased drug exposure
- Sotatercept, or the related molecule luspatercept, may provide a favorable benefit-risk profile for patients with TD or NTD β-thalassemia, addressing a significant unmet need
Domanda 2

• Quale è il meccanismo attraverso cui sotatercept riduce l’eritropoiesi inefficace?
• Pensate che l’efficacia terapeutica sia sufficiente per eliminare le trasfusioni?
WSG of EHA on RED CELLS AND IRON

Agenda:
- Update on EHA Research Roadmap (A. Iolascon)
- Update on the preparation of the Guidelines:
  . Splenectomy (A. Iolascon)
  . HA and pregnancy (A. Taher)
  . Microcytic atypic anemias (P. Aguilar-Martinez)
  . HA iron overload: diagnosis and treatment (MD Cappellini)
- Collaborative Project on treatment of PK deficiency (Zanella A/Mizer K)
- New ENERCA projects: application and update (JV. Corrons, P. Aguilar-Martinez)
- MSc course in Inherited Haemoglobin Disorders (D. Loukopoulos)
- Proposal for future ad-hoc meetings
EHA RESEARCH ROADMAP . Sec. 4 : Anemias and related disorders

• Introduction

• **Table: the best of research roadmap in anemias**
  - epidemiology of anemias in Europe
  - common flow-charts for diagnosis
  - pathogenesis studies of rare inherited anemias to have new therapeutic targets

• enhance clinical trials for new drugs

• use of new technologies for a personalized diagnosis and therapy
Applicazione della NGS allo studio ed alla diagnosi delle anemie rare

Achille Iolascon

Dipartimento Medicina Molecolare e Biotecnologie Mediche
Università degli Studi di Napoli “Federico II”
Genetic Era:
From DNA structure to Complete Sequence

1953 - Discovery of double helix (Watson and Crick)

1975 - Southern blotting

1985 - Description of PCR technology

1978 - First Molecular Diagnosis

1990 - Start of Human Genome Project

1990 - First experiment of Gene Therapy: ADA deficiency correction

2000 - First announcement of decodification of entire human genome
Era Pre-genomica  →  Era Post-genomica
Whole genome sequencing vs Exome-sequencing: diagnosis and discovery

Discovery of “novel” disease genes: sequencing of genomic DNA

- **Whole-genome sequencing (WGS):** characterize entire genomes of any size and complexity

- **Exome sequencing:** sequence protein coding regions, as cost-effective alternative to WGS

- **RNA-Seq:** cDNA generated from RNA allows for mutation analysis in expressed genes
Fanconi anemia (FA) is a rare inherited syndrome characterized by developmental defects, short stature, bone marrow failure, and an increased risk to malignancies. Fifteen genetic subtypes of FA have been identified so far.

Cells derived from FA patients are hypersensitive to chromosomal breakage induced by DNA interstrand cross-linking agents (ICLs) such as mitomycin C (MMC) or diepoxybutane (DEB) and assessment of this cellular hypersensitivity is the classic diagnostic test for FA.

However, the chromosomal breakage test is positive in only ~10% of patients and occasionally the test gives false positive results in other genetic disorders, such as Nijmegen breakage syndrome (NBS; MIM #251260) and Roberts syndrome (RBS, MIM #268300).

*Rosendorff and Bernstein, 1988; Gille et al., 2012*
Fanconi anemia (FA) in Post-genomic era

- A male patient, aged 4 years and 11 months, was born to a healthy non-consanguineous parents;
- The patient had left hand preaxial polydactyly and an irregular hypopigmentation spot on the left back trunk;
- Surgery to correct the thumb deformity was performed when the patient was 6 months of age;
- The patient's development was not officially tested, but was estimated to be within normal range;
- The patient's family history was unremarkable.

### Summary of variations in FA genes revealed by whole exome sequencing in the patient with FA.

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</table>

*: homzygous mutation; √: heterozygous mutation; F: father; M: mother; C: child (patient); # Orphan; *SNP.
Targeted-NGS as diagnostic tool for genetic disorders

- We have not yet reached a point at which routine sequencing of large numbers of whole eukaryotic genomes is feasible
- It is often necessary to select genomic regions of interest and to enrich these regions before sequencing
- There are several enrichment approaches (for example, HaloPlex)

Target re-sequencing facilitates the creation of rapid, accurate and lower cost diagnostic applications
Hereditary hemolytic anemias (HHA)

- Estimated prevalence of HHA: from 1/1,000 to 1/1,000,000
- Differential diagnosis of HHA is often difficult and requires specialized analyses
- A lot of non-specific and overlapping phenotypes between different conditions

Hemolytic anemia

Reticulocyte count

- Normal or reduction
  - Hyporegenerative anemias (HHA)
- Increase
  - Hemolytic Anemias due to red cell Membrane Defects (HAMD)
  - Exclusion of anemias due to:
    - autoimmunity
    - acquired condition

Adapted from Andolfo I, Russo R, Iolascon A. Prosp Ped 2014;44:1-7
Congenital Dyserythropoietic Anemias (CDAs)

- CDAs are mendelian diseases affecting the normal differentiation-proliferation pathway of the erythroid lineage.
- They belong to a subtype of bone marrow failure syndromes characterized by monolineage involvement and morphological abnormalities in erythroid precursor cells.

Erythroid hyperplasia with binuclearity or multinuclearity involving late erythroblasts

- Mild hemolytic anemia (9-10 g/dL)
- Reduced reticulocyte count
- Jaundice
- Splenomegaly
- Hemosiderosis
- Gallstones
- Transfusion dependence (≈ 20%)
Hemolytic Anemias due to red cell Membrane Defects (HAMDs)

- Mild hemolytic anemia (9-10 g/dL)
- Increased reticulocyte count
- Jaundice
- Splenomegaly

- Gallstones

Adapted from Andolfo I, et al. Prosp Ped 2014;44:1-7
Differential diagnosis

HS

- Splenectomy is the standard treatment
- Autosomal dominant (75%)
- Normal or slightly increased sTFR
- Unc. Bilirubin
- LDH
- Haptoglobin
- Hepatosplenomegaly
- RDW

CDA II

- Splenectomy slightly increases Hb level
- Autosomal recessive
- Hemosiderosis
- RDW
- sTFR
Preliminary study
Cohort of patients and causative genes

332 total cases (298 independent pedigrees):
✓ 122 HAMD patients (115 unrelated pedigrees)
✓ 140 CDA patients (118 unrelated families)
✓ 70 patients (65 unrelated families) with unclassified anemia

All patients are included in the International Registry (Napoli, Italy)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene</th>
<th>CCDS</th>
<th>RefSeq</th>
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<th>Amplicon size (bp)</th>
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<td>Chr15:43,015,757-43,029,417</td>
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<td>CCDS13137</td>
<td>NM_006363.4</td>
<td>Chr20:18,488,137-18,542,059</td>
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<td>CDA IV</td>
<td>KLF1</td>
<td>CCDS12285</td>
<td>NM_006563.3</td>
<td>Chr19:12,995,236-12,998,017</td>
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<td>NM_002049.3</td>
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<td>CCDS6121</td>
<td>NM_000037.3</td>
<td>Chr8:41,510,739-41,754,280</td>
<td>17096</td>
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<td>EPB4.1</td>
<td>CCDS331</td>
<td>NM_004437.3</td>
<td>Chr1:29,213,603-29,446,553</td>
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<td>CCDS32100</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>
Preliminary study design

Pilot study

- Selection of 15 patients:
  - 6 with known genotype;
  - 6 with unknown genotype;
  - 1 family

- Panel of 10 causative genes of HS and CDAs (2012-2013)

- Coding regions, UTRs, regulatory regions, 100 bp flanking splice junctions

Inheritance pattern and validation by Sanger sequencing

Diagram:
- Missense nonsense 50%
- Splicing 12%
- Regulatory 2%
- Small del/ins 12%
- Gross del/ins 3%
The major technical challenge of NGS approach is the bioinformatic analyses of massive amounts of data.

**Table I. SureCall categorization**

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category I</td>
<td>Coding variants</td>
</tr>
<tr>
<td>Read Alignment</td>
<td>Any missense mutation, nonsense or frame shift mutation, introduction of a stop codon, results in codon change, results in codon insertion, results in codon change and codon deletion, results in codon change and codon insertion, results in codon deletion, missense mutation of the normal stop codon, mutates in the initiation codon (ATG), deletes nucleotide(s) that lead(s) to a shift of reading frame, deletes exon which results in shift of reading frame, is non-synonymous coding variant in start, is non-synonymous coding variant in stop, is non-synonymous coding variant, is synonymous coding variant, in-frame amino acid insertion/deletion, in-frame exon deletion.</td>
</tr>
<tr>
<td>Adapter Removal</td>
<td>Bam File Generation</td>
</tr>
<tr>
<td>Identify Mutations</td>
<td>Mutation Impact Analysis</td>
</tr>
<tr>
<td>Report Generation</td>
<td>QC Report</td>
</tr>
<tr>
<td>Category II</td>
<td>Splicing variants</td>
</tr>
<tr>
<td>Located within a splice consensus sequence, is splice site donor, is splice site acceptor, alters the sequence at a splice junction, likely to produce a cryptic splice site.</td>
<td></td>
</tr>
<tr>
<td>Category III</td>
<td>Regulatory variants</td>
</tr>
<tr>
<td>Modifies UTR 3', modifies UTR 5', deletes UTR 3', deletes UTR 5', likely to affect transcription.</td>
<td></td>
</tr>
<tr>
<td>Category IV</td>
<td>Intronic variants</td>
</tr>
<tr>
<td>Sequence changes that occur in the intron, is intergenic.</td>
<td></td>
</tr>
</tbody>
</table>

The major technical challenge of NGS approach is the bioinformatic analyses of massive amounts of data.
Variants in clinical report of targeted-NGS-based diagnosis for HHA patients

Filtering variants (1):

- MAF (1000 genomes and EVS)
- Strend bias

Filtering variants (2):

- PolyPhen
- SIFT
- HSF

<table>
<thead>
<tr>
<th>Total variants</th>
<th>62–122</th>
</tr>
</thead>
<tbody>
<tr>
<td>Off-target gene variants</td>
<td>0–2</td>
</tr>
<tr>
<td>Target gene variants</td>
<td>55–105</td>
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<tr>
<td>Intronic and regulatory gene variants</td>
<td>48–92</td>
</tr>
<tr>
<td>Coding gene variants</td>
<td>5–13</td>
</tr>
</tbody>
</table>
Acknowledgements
TRAINING COURSE ON
DIAGNOSIS AND MANAGEMENT
OF VERY RARE RED CELL AND IRON DISORDERS

Lisbon, Portugal
January 29-30, 2016

Chairs: Patricia Aguilar-Martinez, Paola Bianchi, Achille Iolascon, Richard Van Wijk, Alberto Zanella

Topics:
- Disorders of Red Cell Production
- Disorders of Red Cell Survival – Hereditary Haemolytic Anaemias
- Disorders of Red Cell Survival – Acquired Haemolytic Anaemias
- Diagnosis and Treatment of Very Rare Anaemias

To register and for further information: www.esh.org
Suragani et al. Nat Med, 4, 408-14, 2014