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CLINICAL EXOME APPROACH IN FAMILIES WITH
HEREDITARY NEUROMUSCULAR DISEASES

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*C'è un'evidenza prima e uno stupore del quale è carico
l'atteggiamento del vero ricercatore: la meraviglia della
presenza mi attira, ecco come scatta in me la ricerca.*
L.Giussani

*A Lory e Fra,
perse durante questo cammino*

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INTRODUCTION

«The *Human Genome Project* (HGP) was one of the great feats of exploration in history - an inward voyage of discovery rather than an outward exploration of the planet or the cosmos; an international research effort to sequence and map all of the genes of members of our species, *Homo sapiens*».

National Institute of Health

1. Next Generation Sequencing and the “omics” revolution

The HGP project was completed in April 2003, with a cost of \$3 billion dollars and the contribution of six different nations. The HGP gave us the ability, for the first time, to read into the genetic project of human being and to know his complete genomic sequence.

The work on interpretation of genome data is still in its initial stage and it is possible to foresee many future benefits in various fields, as for example: a) in the field of molecular - medicine it will improve diagnosis of diseases, early detection of genetic diseases and therapy. Suitable treatment can be prescribed and drug design can be undertaken because genetics will disclose the differences between the individuals. Thus, treatment can be more focused and effective based on individual need; b) in the field of forensic sciences DNA can be used in identifying war victim's and it can provide full proof results in ascertaining disputed parentage so that criminal justice can be more effective; c) in the field of agriculture and livestock breeding knowing information of plant and animal genome will help us to create stronger and more disease resistant plants and animals. Moreover it will facilitate the development of bio-pesticide and edible vaccines incorporated into food products.

The Human Genome Project was accomplished with the “first generation” DNA sequencing, known as Sanger sequencing (the chain terminator method), developed in 1975 by Edward Sanger (Sanger et al., 1977; Grada and Weinbrecht, 2013). This technique was used to obtain the first consensus sequence of the human genome in 2001 (Lander et al., 2001; Venter et al., 2001) and the first individual human diploid sequence (J. Craig Venter) in 2007 (Levy et al., 2007).

In order to sequence longer section of DNA, a new approach called shotgun sequencing was developed during HGP. In this technique, genomic DNA was enzymatically or mechanically broken down into smaller fragments and cloned into sequencing vectors in which cloned DNA fragments can be sequenced singularly. The complete sequence of a long DNA fragment can be generated by these methods through alignment and reassembly of sequence fragments based on

partial sequence overlaps. Shotgun sequencing was a significant advantage for HGP, and allowing the sequencing of the entire human genome possible.

Shortly after, was completely sequenced the second individual genome (of James D. Watson) using next-generation technology, adapted from shotgun sequencing method, which marked the first human genome sequenced with new NGS technology (Venter et al., 2003; Margulies et al., 2005; Shendure et al., 2005; Wheeler et al., 2008).

A core philosophy of next generation sequencing is to use DNA synthesis or ligation process to read through many different DNA templates in parallel. Therefore, NGS is able to read DNA templates in a highly parallel manner to generate massive amounts of sequencing data, but the read length for each DNA template is relatively short (35-500 bp) compared to traditional Sanger sequencing (1000-1200 bp). This massively parallel sequencing, in which millions of fragments of DNA are sequenced in unison, represents a very high-throughput technology; in fact an entire genome can be read in less than one day (Fuller et al., 2009).

The simultaneous evolution of bioinformatics and computers with high-efficiency, implemented during the realization of HGP, enabled the advent of platforms for the next generation sequencing (NGS) more efficient and cost-effective.

NGS can be considered an evolution of microarray technology and sequencing. In fact, it can provide information that partially overlap with data made available through the use of microarray technology. However, NGS possesses several advantages over current microarray platforms, as for example a better sensitivity, and a lower background noise. NGS is also a versatile technology that permits the detection of sequence variants at many levels: of the gene (re-sequencing of target regions), the exome, or the whole genome. Moreover, NGS can be used to analyse many aspects of the genome: gene expression (or transcriptome), methylation status (or methylome), and chromatin structure (Mori et al., 2013).

In conclusion NGS technologies have increased the speed and throughput capacities of DNA sequencing and, as a result, dramatically reduced overall sequencing costs (Mardis, 2006, 2009; Schuster, 2008; Shendure and Ji, 2008; Ng et al., 2009; Tucker et al., 2009; Metzker, 2010); consequently the cost of genome sequencing dropped down from ~3 billion dollars during HGP to few thousand dollars in the last years (Gullapalli et al., 2012).

2. Next Generation Sequencing platforms

All NGS platforms on the market are characterized by the ability to sequence in massively and parallel way clonally amplified or single DNA molecules.

NGS sequencing is operated through the repetition of cycles of nucleotide extension or ligation of oligonucleotides. Peculiar steps that characterize the different platforms on the market are: chemistry of sequencing and the acquisition of the images.

The platforms have a similar workflow that consists of template preparation, sequencing, imaging, and data analysis (Metzker, 2010).

The three major NGS methods routinely used in many laboratories today include: 1) the Roche GS-FLX 454 Genome Sequencer (<http://www.454.com>), 2) the Illumina/Solexa Genome Analyzer (<http://www.illumina.com>), 3) the Applied Biosystems SOLiD™ System (<http://marketing.appliedbiosystems.com>).

Other three massively parallel technologies that have been introduced more recently include: the Polonator (Dover/Harvard), the HeliScope Single Molecule Sequencer technology (Helicos, Cambridge, MA, USA), (Shendure et al., 2005; Mardis, 2008) and the Ion Semiconductor (Torrent Ion Sequencing).

The single molecule real time (SMRT) (Pacific Biosciences) and Nanopore Sequencing are other two newly introduced technologies that are based on the sequencing of single molecules (Shendure and Ji, 2008). The main characteristics of major platforms are summarized in table 1.

Company	Platform	Amplification	Sequencing	Read length	Throughput/ time per run	Dominant error type	Overall error rate
Roche/454 Life Sciences	GS FLX Titanium XL+	Emulsion	Pyrosequencing	Up to 1 kb	700 Mb/23 h	Indel	0.5%
	GS FLX Titanium XLR70	PCR		Up to 600 bp	450 Mb/10 h		
	GS Junior HiSeq 2000			~400 bp 36–100 bp	35 Mb/10 h 105–600 Gb/2–11 days		
Illumina	Genome Analyzer IIx	Bridge PCR	Sequencing-by-synthesis with reversible terminator	35–150 bp	10–95 Gb/2–14 days	Substitution	0.2%
	MiSeq			36–250 bp	540 Mb–8.5 Gb/4–39 h		
Life Technologies/ Applied Biosystems	5500xl SOLiD™ system SOLiD™ 4 system	Emulsion PCR	Sequencing by ligation	35–75 bp 25–50 bp	10–15 Gb/day 25–100 Gb/3.5–16 days	Substitution	0.1%
Life Technologies/Ion Torrent	Ion Proton™ sequencer (Proton I chip)	Emulsion PCR	Ion semiconductor sequencing	Up to 200 bp	Up to 10 Gb/2–4 h	Indel	1%
	Ion PGM™ sequencer (318 chip)			35–200 bp	300 Mb–1 Gb/0.9–4.5 h		
Helicos BioSciences	HeliScope™ single molecule sequencer	NONE	Single molecule sequencing	25–55 bp	21–35 Gb/8 days	Deletion	5%
Pacific Biosciences	PacBio RS	NONE	Single molecule sequencing	250 bp–10 kb	NA	Indel	15%

Table 1. Overview of major next generation sequencing platforms (Xuan et al., 2013)

These NGS instruments generate different base read lengths, different error rates, and different error profiles respect to Sanger sequencing data and to each other.

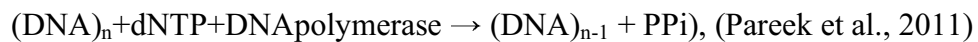
2.1 The “second generation”

The second generation sequencing technologies share a common step of DNA isolation followed by the creation of single stranded DNA libraries performed by its fragmentation using various techniques (Gullapalli et al., 2012). The fragmentation of genomic DNA produces fragments of small dimensions which vary from 100 to 500 base pairs (bp) in relation to the platform; then adapters are added to these fragments, and can be used for the next amplification step of the genomic fragments.

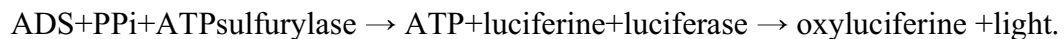
- *454 Pyrosequencing*

The 454 Life Sciences (Branford, CT, USA) was the first next generation sequencing technology that was commercially available (in 2005) (later acquired by Roche) and was also the first NGS technology used to sequence a complete human genome, that of Dr. James Watson (Scheibye-Alsing et al., 2009). This method is based on using a sequencing technique called “pyrosequencing”, which was first introduced by PålNyren and Mostafa Ronaghi at Stockholm’s Royal Institute of Technology in 1996.

The pyrosequencing is a bioluminescence method that uses the sequencing-by-synthesis concept which is different from the Sanger method that is based on chain termination with dideoxynucleotides. During the incorporation of each nucleotide the pyrophosphate is released as the following reaction:

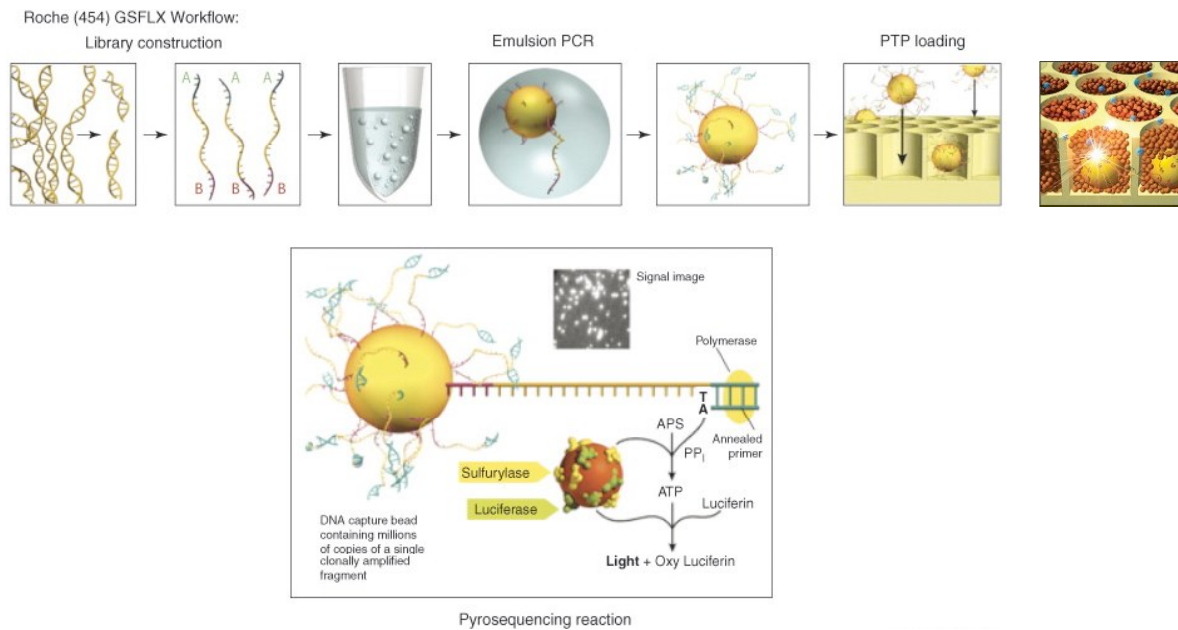


The pyrophosphate in presence of adenosine 5’-phosphosulfate can be converted into ATP by enzyme ATP sulfurylase. Subsequently, a chemiluminescent enzyme, luciferase, using ATP as substrate, convert luciferine to oxyluciferine and light emitted can be detected by a charged coupled device (CCD) camera. The reaction is the following:



The individual molecules of ssDNA ligated with short adaptors at their blunt ends (3’ and 5’ ends), (fig.1 a/b) are mixed with a population of small (28-µm) streptavidin-coated beads covered by oligonucleotides complementary to one of the ligated adaptors. Fragments are then hybridized to their corresponding bead in a way that each bead is associated with a single-unique molecule of DNA.

a)



b)

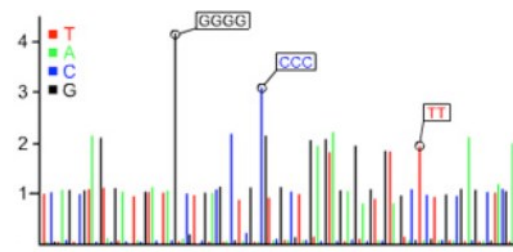


Figure 1. Schematic representation of 454 pyrosequencing technology. a) Construction of the library and amplification of single fragments DNA forming “clone beads”; the beads are then deposited into the “picotiter plate” wells. In the pyrosequencing method nucleotide incorporation is determined by the detection of light emitted by the enzymatic activity of luciferase enzyme in presence of ATP and luciferine. b) Typical flowgram of 454 that show the order of nucleotide sequenced

Amplification of DNA fragments are performed through emulsion PCR, described for the first time by Tawfik and Griffiths (Tawfik and Griffiths, 1998), in which all the required reagents are provided with droplets of water in oil mixture. Fragment/bead complexes are encapsulated by vigorous vortexing into these micelles that acts as a micro-reactor for every single bead. Fragment amplification is required for intensifying the light signal which is necessary for precise detection by CCD camera. At the end of the process, approximately 1 million copies of each amplified DNA fragment is present on the surface of each bead. After breaking the emulsions, the beads that were incubated with DNA polymerase, can be loaded into PicoTiterPlate device and every single bead will be placed in a well with the dimension that can contain only one bead.

Smaller beads (1- μ m) carrying sulfurylase and leuciferase enzyme will fill the wells and this will help the beads to deposit at the bottom (Nowrousian, 2010).

During the pyrosequencing process, as the polymerase synthesizes the DNA strand, the light emitted from phosphate molecules during nucleotide incorporation is recorded. The amount of light produced is proportional to the number of nucleotides incorporated.

The maximum length of sequenced reads was 450 bp and now it has been increased to about 700 bp. The 454 Sequencer has the longest short reads among all the NGS platforms; and generates ~400-600 Mb of sequence reads per run.

However, error-prone raw data sequence, especially associated with insertion-deletions, is a major concern. Raw base accuracy reported by Roche is very good (over 99%).

- *Illumina (Solexa) Genome Analyzer*

The Genome Analyzer was the second platform that reaches the market, and currently is the most widely used system. Initially the read length was very short (36 bp or less), but now it is up to 100 bp (Kircher and Kelso, 2010). The first step is the creation of DNA library of adaptor-flanked fragments (fig.2a). The amplification step is performed on a “flowcell” (a solid surface support), on which are attached both forward and reverse primers that are complementary to adaptor’s sequences. Template amplification is carried out by a method called “bridge PCR”. The name refers to the fact that during the annealing step, the extension product from one bound primer forms a bridge to the other bound primer and its complementary strand is synthesized.

The amplified fragments will form clusters, each of which will contain approximately 1 million copies of the initial fragment. Moreover, because in each cluster are represented both forward as well as reverse strands of the original sequence, one of the strands must be removed before initiating the sequencing process.

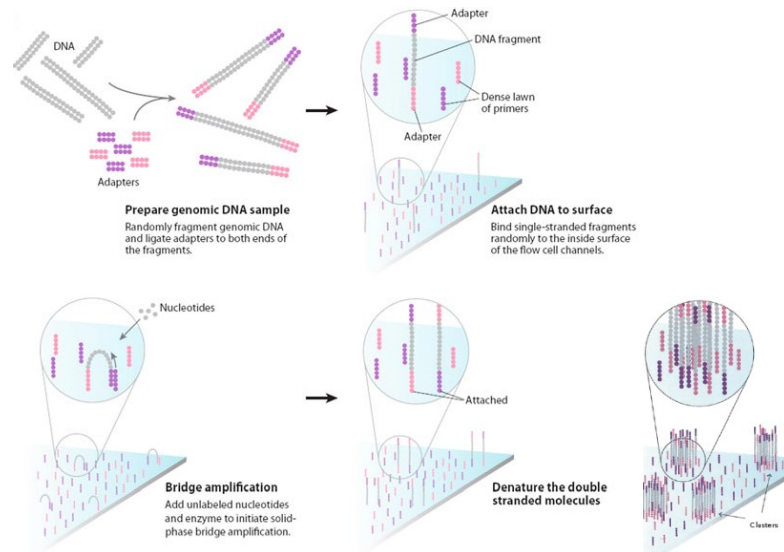
Illumina system adopts a sequencing-by-synthesis approach based on cyclic reversible terminator system (CRT). In this method, four reversible blocked nucleotides (3'-OH is chemically blocked) are added to the reaction so that each of incorporation is a unique event.

Each cycle of sequencing includes: incorporation of the nucleotide, acquisition of the fluorescence and cutting of the nucleotide.

All the four nucleotides are added simultaneously to the flow cell channels along with DNA polymerase that incorporates the modified nucleotides into the fragments.

An imaging acquisition step follows each incorporation step. After each imaging step, the 3'-OH blocking groups are chemically removed by a washing step to prepare each strand for the next incorporation by DNA polymerase (fig.2b).

a)



b)

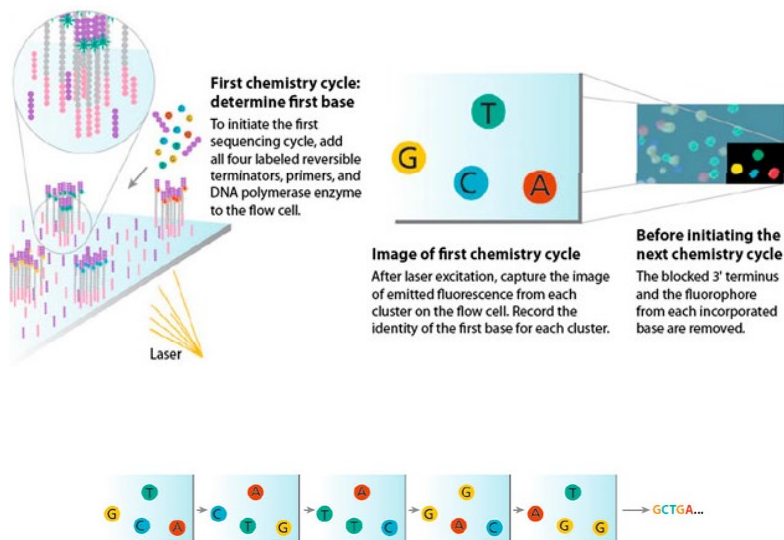


Figure 2. Schematic workflow of the Illumina sequencing-by-synthesis technology. a) Clonal amplification of DNA adaptor-flanked fragments with bridge amplification; after denaturation ssDNA fragments produced are used for the sequencing step. b) Sequencing and base detection. After incorporation of 3' blocked nucleotides image is detected and the blocked nucleotides are removed with the fluorescent dyes to restart another sequencing cycle

The performance of Illumina sequencer, GA, was improved from 1G/run to 85G/run in the GAII series, which was released in 2009. In early 2010, Illumina launched HiSeq 2000, which adopts the same sequencing strategy with GA. Currently its output is improved to 600G per run and is capable to produce single reads of 2x100 base pairs (pair-end reads). The raw base accuracy is greater than 99.5%. In the 2011, MiSeq, a bench-top Illumina sequencer, was

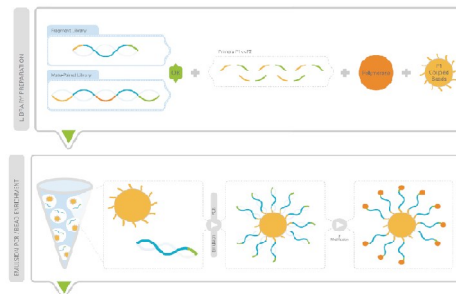
introduced: it shared most technology with HiSeq and it is mainly used for amplicon and bacterial sample sequencing (Liu et al., 2012).

- *Applied Biosystems SOLiD Sequencing*

The SOLiD system became commercially available in late 2007 and was developed by J.S. and colleagues in collaboration with McKernan and colleagues at Agencourt Personal Genomics.

The method is based on emulsion PCR and uses sequencing-by-ligation approach. After the construction of DNA library (adaptor-flanked DNA fragments), the clonal amplification is performed through small magnetic beads (1- μ m in diameter) to which DNA fragments are bound. In this way each bead carries an individual fragment (fig. 3a).

a)



b)

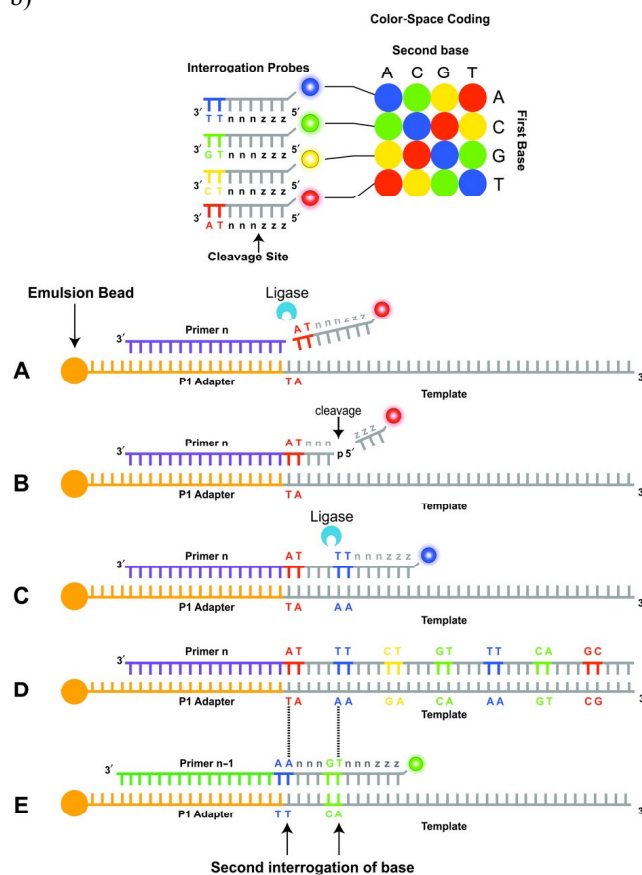


Figure 3. Schematic overview of SOLiD sequencing-by-ligation technology.

a) Library preparation and clonal amplification of DNA fragments on the beads; deposition of 3' modified beads onto a glass slide;
 b) sequencing by ligation process.

(Voelkerding et al., 2009)

After the amplification, the “beads clones” are selectively isolated and tethered to a “flow cell” through 3’ modification of DNA strands. This flow cell is a microscopic slide that can be exposed to liquids. On the SOLiD analyser a universal sequencing primer is annealed with the template-beads; in fact it is complementary to the common adaptor in each fragment.

Differently from other platforms in which base detection of DNA fragments is performed through PCR, in the SOLiD platform this is accomplished by sequencing-by-ligation approach.

Together with universal primer, a ligase and a large pool of fluorescently labelled octamers, with 4 distinct fluorescent dyes, are included. Each octamer has a di-nucleotide sequence combination (out of 16 possible combinations), which in different versions of ABI-SOLiD sequencing chemistry is different (e.g., in ABI-SOLiD v.2.0 position 1 and 2, and in the original version, bases 4 and 5 are utilized). The rest of the bases are degenerated (nnzzz). Each octamer holds one of four fluorescent dyes that identify a two-base combination in that octamer. Each cycle of ligation is composed of 5 rounds (fig 3b);

1. Annealing of universal primer with “n” nucleotides to each DNA fragment; then a pool of octamers with specific dinucleotide composition is added. If this dinucleotide matches with template, it can be hybridized and ligated (in presence of enzyme ligase);
2. Image acquisition: the specific fluorescence is detected, which corresponds to a specific dinucleotide combination. Each fluorescent dye is in correlation with four di-base combination;
3. Washing: to eliminate probes that are not linked to template;
4. Cutting: the last three bases (i.e., 6-7-8) of octamer along with the fluorescent dye are chemically removed, leaving a free 5’-phosphate group available for further reactions. This process is repeated for 7 cycles and yields a 35bp read. Additional cycles can be added to extend the read length;
5. The synthesized strand is removed by denaturation and new primer is hybridized offset by one base and ligation cycles are repeated. This primer’ reset process is repeated for 5 rounds providing dual measurements of each base.

SOLiD analyzer has a read length up to 50 bp and can produce 80-100 Gbp of mapped sequences per run. The latest model, 5500xl solid system (previously known as SOLiD4) can generate over 2.4 billion reads per run with a raw base accuracy of 99.94% due to its 2-base encoding mechanism. This instrument is unique in that it can process two slides at a time; one slide is receiving reagents while the other is being imaged.

- *Ion Torrent*

The Ion Torrent platform uses the ion semiconductor sequencing, a method that is based on the capture of a hydrogen ions that are released during the formation of a covalent bond between nucleotides during the polymerization of DNA (fig. 4).

DNA is fragmented, ligated to adapters, and adaptor-ligated libraries are clonally amplified onto beads. Template-beads are enriched through a magnetic-bead-based process.

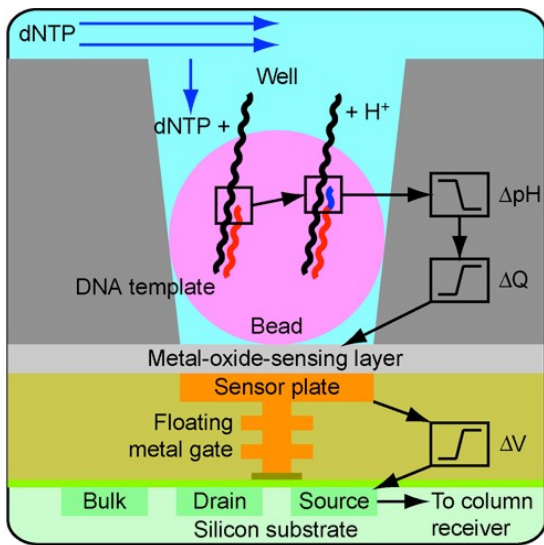


Figure 4. A simplified drawing of ION TORRENT sequencing technology. When the nucleotides (dNTPs) are incorporated on the growing DNA strands releasing of protons (H^+) causes a pH change of the well (ΔpH). This induces a change in surface potential of the metal-oxide-sensing layer, and a change in potential (ΔV) of the underlying field-effect transistor

The beads are flooded in a stepwise fashion during an automated run with a single species of deoxyribonucleotide triphosphate (dNTP) in a microwell. The release of the proton produces a shift in the pH of the surrounding solution proportional to the number of nucleotides incorporated in the flow (0.02 pH units per single base incorporation). The change in pH is detected by the Ion-sensitive field effect transistor (ISFET), located on the bottom of each well.

This change is recorded as a potential change (ΔV) by an ion sensor layer that indicates the nucleotide incorporation event (Hui, 2012). After the flow of each nucleotide, a wash is used to ensure nucleotides do not remain in the well. This technology is characterized by a transformation of a

chemical signal in a digital signal and chemistry of sequencing differs from the other platforms in that no enzymatic reaction, no fluorescence, no optic and no light are used to determine the sequence of DNA. The size of DNA fragments now has been improved to 400 bp.

2.2 The “third generation”

The third generation instruments differ from the 2nd generation instruments in that the initial DNA amplification step is unnecessary and in a marked reduction of costs for sequencing. The “third generation” sequencers consist of HelicosHeliscope, Pacific Biosciences SMRT, and Oxford Nanopore.

- *Heliscope*

The HelicosHeliScope platform is the first single molecular sequencing technology that was introduced (Wash and Image, 2008). The characteristic of this technology is its ability to sequence single DNA molecules without amplification, a process defined “single-molecule real-time (SMRT) DNA sequencing”. The Helicos platform uses the same technique of the Illumina, sequencing-by-synthesis and reversible terminator nucleotides, but the difference is that all the four nucleotides are labeled with the same fluorochrome and are distributed in the sequencer with a precise hierarchical order.

In fact, after shearing a single DNA molecule into small pieces (100-200bp) and poly-A-tailing, these fragments are bound to the surface of the flow cell using complementarity with poly-T already attached on the surface (fig.5a). A mixture of one color-labelled nucleotide and polymerase flowed into the surface; the polymerase will add the nucleotide if there will be complementarity between the added base and the first position of attached DNA fragment. A camera scans the entire surface and the image is taken from all bound fragments containing the incorporated labelled nucleotides. Only when the label is cleaved off from the attached base, the next cycle can be performed. The incorporation can arrive to 25-45 bases.

- *Nanopore Sequencing*

Using Nanopore technology, DNA sequencing can be performed by threading it through a microscopic pore in a membrane. This methodology was introduced by Oxford Nanopore Technology and seems to be able sequencing of the entire human genome to \$1.000 in the near future. The technique is based on the modulation or fluctuation of the ionic current by the passage of a DNA strand through the pore (fig.5b).

The translocation of each base through the pore causes a decrease in current intensity, which is specific for each kind of base and it is possible to identify the specific nucleotide by the measurement of this current.

However the registration step presents some problems, as a result of the high speed of passage of DNA strand. Recent works show that this problem may be overcome (Clarke et al., 2009).

Another version of Nanopore technology has been introduced by McNally (McNally et al., 2010). As shown by the author the solid state sub-5 nm pores can be parallelized in the form of a very condensed array. An important biochemical step is represented by the conversion of each base of the sequence into an oligonucleotide that can be hybridized with a molecular beacon. The threading of the beacon through a nanopore allows detecting the optical signal. The signals are projected on a wide field imaging screen; using an EM-CCD camera is possible.

- *SMRT*

SMRT technology harnesses the natural process of DNA replication. DNA sequencing is performed on SMRT Cells, each patterned with 150,000 zero-mode waveguides or ZMWs (fig.5c).

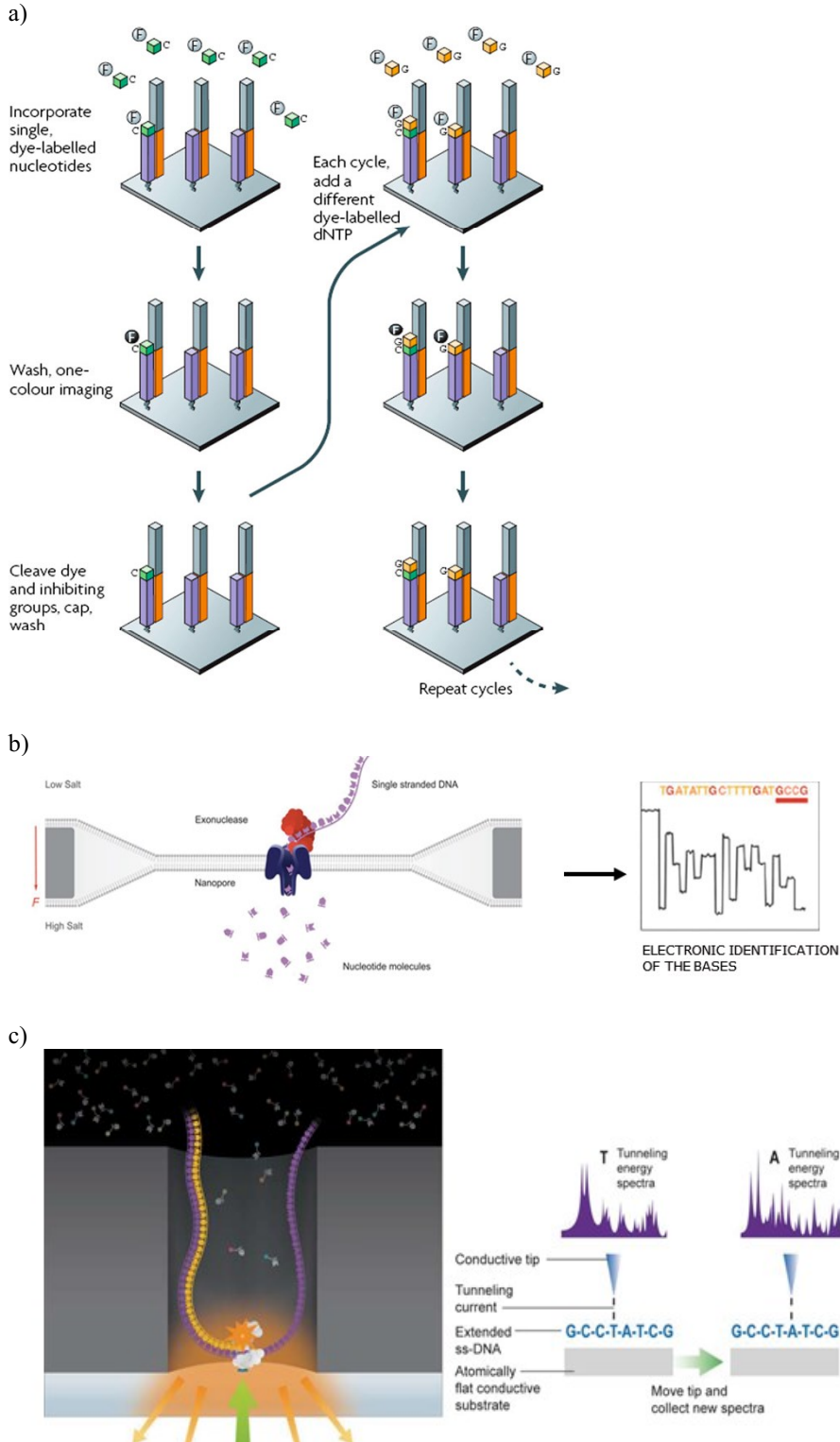


Figure 5. Third generation sequencing core methodology.

a) In the Helicos platform DNA molecules are bound to the surface of the flow cell; when DNA polymerase and labeled single base flow into the surface, nucleotide incorporation can be added if complementarities exist with the first position of attached DNA fragments. Then a camera scans the entire surface (one color detection system) and, after labeling dye and inhibitor are removed, can start another cycle of sequencing.

b) In Nanopore system a translocation of DNA molecule occurs through the pore and each nucleotide can be identified by the way it affect ion current across the pore.

c) In ZMW system a DNA polymerase is confined in a zero-mode waveguide and base addition is measured with fluorescence detection of gamma-labeled phosphonucleotides

(Schadt et al., 2010; Masoudi-Nejad et al., 2013)

Single polymerases are immobilized in the ZMWs, which provide the windows to observe DNA sequencing in real-time. Phospholinked nucleotides are introduced into the chamber. Each of the four nucleotides is labeled with a specific fluorophore. As a base is held in the detection volume, a pulse light is produced. During incorporation the phosphate chain is cleaved and the fluorophore is released.

2.3 Data Analysis

The NGS experiments generate unprecedented amounts of information and represent a big challenge for their management, storage and analysis. All platforms follow a “Data-pipeline system”, an interconnected system with operative capacity for storage, handling and processing the data. The typical output of NGS generated is in the order of Terabyte (Tb) (454 GS FLX -15 GB, Illumina-1TB, SOLiD15 TB) during a single run.

The raw data is usually in the form of a text file, which contains “reads” which are short sequences of DNA letters corresponding to the nucleotides incorporated during the process of sequencing. Each data output file contains millions of these “reads” in each data file.

The task of data-pipeline-system is the conversion of the luminescence or fluorescence images acquired, in a list of billions of short nucleotide sequences (25 to 400 bp) called “reads”, a process called “base calling”. Once the raw sequence data is obtained, the computationally intensive step of read mapping will be performed. The mapping software then attempts to “map” the individual sequence NGS “reads” onto a reference genome sequence available from online genome databases. This process is known as reference mapping. Next generation sequencing data is usually a text (or binary) file in the FASTQ format (Cock et al., 2010).

The molecular reliability of NGS is based on three criteria: depth of coverage, heterogeneity, and accuracy of sequencing. Depth of coverage indicates the number of times that each nucleotide is sequenced (e.g. $5\times$ coverage, means that each nucleotide of the target region has been sequenced five times). Heterogeneity is a measure of uneven sequencing depth of coverage along the length of the expressed region. Finally, the accuracy of sequencing is indicated by the quality of the base calling (i.e., the quality scores assigned to each base call in automated sequencer trace, known as *phred* scores), (Mori et al., 2013).

Through the assignment of a “quality score” (by platform specific algorithm) it is possible to evaluate the associated error probability to each nucleotide and a series of image’s parameters such as intensity, ground noise and aspecific signals.

The “quality score” represents an important tool that eliminates from the process the bases or “reads” that do not pass the appropriate parameters, improving in this way the accuracy of the next “pipeline” (i.e. the alignment of the reads with reference sequence and base variant annotation (“variant calls”), (Li et al., 2008).

The next alignment “pipeline” requires “reads” that overcomes 30 bp for a correct efficiency. In fact, only 90% of the human genome can be aligned uniquely with reads of 30 bp (Whiteford et al., 2005).

A further limitation to the alignment is given by the repetitive sequences in the genome; the solution consists of the assignment of the “reads” to multiple locations in the reference genome or in the creation of gaps (“gaps”) in the alignment. The error rate associated with the next generation technology seems more than the traditional Sanger method. However, the accuracy of the NGS sequencing platforms is ensured by a repeated and massive reading of each genic fragment, which determines the “coverage” of the genome, given by the sum of overlapping “reads”.

Coverage represent an essential parameter for the NGS analysis, because an inaccurate “coverage” (with value lower than 20-50 times in relation to the platform) can determine the presence of false negative in heterozygous samples to detect a “true” nucleotide change (Wheeler et al., 2008). Many free bioinformatics tools and software packages are available on-line for the analysis of the sequence data (Gogol-Döring and Chen, 2012).

At the end of the variants annotation process, the multitude of changes identified are subjected to the application of several filters to reduce the number of candidates. The most commonly filters applied are based on:

- patterns of inheritance (autosomal/X-linked; dominant/recessive);
- allele frequency of the variation in the population (for rare or very rare diseases the allele frequency should be very low or even 0);
- sharing the same gene variation(s) in affected sibs;
- sharing the same variation(s) in patients belonging to different but well characterized families;
- excluding the already known variants through the use of public databases (dbSNPs; 1000 genomes);
- prediction of the functional effects of the changes through the use of *in silico* tools (as SIFT, POLYPHEN-2, ANNOVAR, MUTATION TASTER, VarScan, ESE FINDER, SNPeff, PANTHER, ALAMUT, Mutpred, SeattleSeq annotation, Variant Sequence Analyser).
- SANGER validation and segregation analysis in the family

2.4 NGS applications

The applications of next-generation technologies range from the study of the entire genome to the transcriptome. Most common diseases and quantitative traits in human populations have a complex genetic basis. The identification of genetic rare variants that underlie susceptibility to common diseases and traits has been the main goal of NGS.

The development of international projects was aimed to sequencing thousands of human genomes that will allow the characterization and the cataloguing of human genetic variation to an unprecedented level. The 1000 Genomes project made great progress to reach this goal developing a more detailed catalogue of genetic variants with frequencies down to 1% in multiple human populations (Durbin et al., 2010; 1000 Genomes Project Consortium, 2010). In the recent years, the UK10K project started with the aim to explore the DNA sequence at an order of magnitude deeper than the 1000 Genomes Project for Europe (carrying out genome-wide sequencing of 4,000 samples), to associate genetic variation with phenotypic traits, to uncover rare variants contributing to disease (sequence 6,000 exomes of extreme phenotypes of specific conditions) and to provide a sequence variation resource for future studies through a genotype/phenotype resource that will be an order of magnitude deeper than the genetic-only 1000 Genomes Project. The data generated by UK10K is an extraordinary possibility for research into human genetics, and it is hoped that the discovery of rare and low frequency disease-causing variants will lead to further insight into the diagnosis and treatment of disease (www.uk10k.org/consortium.html).

In late 2012 it was launched the 100,000 Genomes Project with the aim to sequence 100,000 whole genomes from NHS patients by 2017. The Genomics England, a company wholly owned and funded by the Department of Health, was set up to deliver this flagship and will focus on patients with a rare disease and their families and patients with cancer.

The first samples for sequencing are being taken from patients living in England (<http://www.genomicsengland.co.uk/>).

Another important player in this field is Beijing Genomics Institute (BGI) with their “1000 Plant and Animal Reference Genomes Project” and “Ten Thousand Microbial genomes project”. Considerable efforts have also been invested in the analysis of genomes from endangered or extinct species like panda, mammoth and early humans. In 2009, it was established the Genome 10K Project by a consortium of biologists and genome scientists determined to facilitate the sequencing and analysis of the complete genomes of 10,000 vertebrate species, with the scope to understand how complex animal life evolved through changes in DNA and use this knowledge to become better stewards of the planet. Further, 100K Genome Project was launched in July 2012.

It aims to sequence the genomes of 100,000 infectious microorganisms and eventually speed up the diagnosis of foodborne illnesses (<http://100kgenome.vetmed.ucdavis.edu>).

Another most important field is biomarkers discovery. In fact with the advent of high-throughput technology it is possible to obtain markers information from genomic, transcriptomic, proteomic, metabolomics and modern imaging (Ferlini et al., 2013). In particular, genomic biomarkers include both DNA and RNA determinants, such as polymorphism or allele variations, which could be related to disease susceptibility, disease progression or therapeutic response (Scotton et al., 2014). A further interesting application is “personalized biomarkers”, recently studied in oncology (tumor-specific biomarkers), (Leary et al., 2010; Woollard et al., 2011). Biomarkers are very useful as diagnostic and prognostic tools (for anticipating the severity of the disease course), and as predictive/therapeutic (for defining pharmacokinetic and pharmacodynamics action of drugs and drug response mechanism), (Scotton et al., 2014).

One of the main topics of NGS concerns the analysis of the single nucleotide polymorphisms (SNPs) or single nucleotide variants (SNVs), small insertions and deletions (indels, 1-1000 bp) and structural and genomic variants (>1000bp) comprising large-scale amplifications, deletions, insertions, translocations or inversions (Xi et al., 2010; Zhang et al., 2011).

NGS applications are also concerning DNA methylation; it is a major form of epigenetic modification and plays essential roles in physiology and disease processes. In the human genome, about 80% of cytosines in the 56 million CpG sites are methylated to 5-methylcytosines. The methylation of DNA, through conversion of cytosine of CGs in 5-methyl cytosine in the promoter region, influences gene expression and cancer development. NGS methods have helped in deciphering the methylation profile of the genome (Zhang and Jeltsch, 2010).

The analysis of the transcriptome also concerns the RNA-seq and small RNAs analysis. RNA-Seq refers to sequencing the complex heterogeneity of transcripts that are present in the organism. RNA-Seq has resulted in the production of large amount of new data: for example, finding novel spliced junctions, antisense regulation mode of action, or intragenic expression (Tarazona et al., 2011). “Small” RNAs and other non-coding RNAs sequencing is able to accurate detection and quantification of rare species, discovery novel microRNAs targets and differential expression analysis of known microRNAs. For example a comprehensive study of miRNA in acute myeloid leukemia discovered novel differentially expressed miRNA (Ramsigh et al., 2010; Woollard et al., 2011).

Two other most important applications are Chip-Seq and RIP-seq. The first is used to map a specific DNA binding sequence to a reference genome. To obtain DNA sequence, at first

protein-bound DNA is captured through an interaction between an antibody and protein target. After digestion with DNase to remove DNA not bound, the sequence that is bound to protein is separated, sequenced and matched to the reference. RIP-seq, is a technique analogue of Chip-seq, but it is performed on RNA, instead of DNA, and involves a nuclear or cytoplasmic protein. NGS allows at performing the sequencing of entire genomes, from microorganism to human species. In particular the ability of some platforms to sequence longest “reads”, facilitates the “*de novo* assembly” in the absence of a reference genome.

Another important application is genomic re-sequencing of target regions to identify polymorphisms and mutations in cancer genes or in human genomic regions involved in genetic diseases by linkage or genome wide association studies.

Moreover NGS is applied to a field called meta-genomics. Meta-genomics is used to understand all the genetic information about all the organisms that live together in a certain ecosystem. Examples of metagenomics studies include analysis of ocean and soil microbial populations, the characterization of the micro-flora present in the human oral cavity, and in the gut of obese and thin (Hugenholtz and Tyson, 2008).

3. Whole Exome Sequencing in human diseases

Exome sequencing is a powerful tool for dissecting the genetic basis of diseases and traits that have proved to be intractable to conventional gene-discovery strategies, and provides important knowledge about disease mechanisms, biological pathways and potential therapeutic targets. Interrogation of exome sequencing data for pathogenic variants in known genes associated with a specific disease represent the purpose of clinical exome approach. To date this approach is a very useful application in mendelian disorders such as identification of some known rare genetic modifiers.

The whole exome sequencing represents a very powerful tool to identify causal gene (gene discovery) underlying monogenic disease, in particular for patients with clinical diagnoses of heterogenous genetic conditions and for patients with no specific or unusual disease presentation, and finally for discovery of possible genetic cause of the common complex diseases.

Whole exome sequencing (WES) was first developed in 2009. It is a technique to selectively capture and sequence the coding regions of all annotated protein-coding genes. Coupled with next generation sequencing (NGS) platforms, it enables the analysis of functional regions of the human genomes with unprecedented efficiency. Since its first reported application WES has emerged as a powerful tool for elucidating genetic variants underlying human diseases.

The exome represents less than 1% (3×10^7 base pairs) of the human genome, but it is estimated that it contains ~85% of known disease-causing variants. For this reason whole-exome sequencing represents a great scientific tool to uncover the causes of a large number of rare genetic disorders (mainly monogenic) as well as predisposing variants in common diseases and cancers and it is a cost-effective alternative to whole-genome sequencing (Rabbani et al., 2014; Teer and Mullikin 2010).

On the basis of the disease being studied and its mode of inheritance, a specific strategy can be applied to reach the possible causing variants. For Marian and Belmont (Marian and Belmont, 2011) the variants can be divided into five classes in order of strength of the evidence for causality, as follows:

1. Disease-causing;
2. Likely disease-causing;
3. Disease associated;
4. Functional (including insertions, deletions, nonsense variants, splice variants and copy number variation) but not associated with a disease and are suspected to cause a disease;
5. Unknown biological function.

The clinical significance follows a gradient being the highest for disease-causing variants (category 1) and negligible for variants with unknown biological function (category 5). The frequency of the alleles, in general, is rare for disease-causing variants and common for the variants that are not known to carry biological significance (Marian, 2012; Rabbani et al., 2014).

The first successful application of exome sequencing took place in 2010 with the discovery of *DHODH* gene encoding for a key enzyme in the pyrimidine *de novo* biosynthesis pathway, for a rare mendelian disorder of unknown cause, Miller syndrome (Ng et al., 2010).

Shortly thereafter, the same group identified *MLL2* gene as causative for Kabuki syndrome (Ng et al., 2010). From 2010 until now a large amount of studies have identified new pathogenic mutations, using exome sequencing in a small number of individuals affected, in various types of diseases including cases of neuropathy, poikiloderma associated with neutropenia, exudative vitreo-retinopathy family immunological disorders, intellectual disorders, cancer predisposition (Lin et al., 2012).

WES has led to discover causative mutations in diseases characterized by high phenotypic heterogeneity, a field where the traditional approach of linkage analysis results to be more difficult, for identification of phenotypic severity biomarkers, and development of personalized therapies in the field of pharmacogenomics.

Moreover there is a considerable interest in the use of next generation sequencing to help diagnosis of unidentified genetic conditions, but it is difficult to predict the success rate in a clinical setting that includes patients with a broad range of phenotypic presentations. In fact even though there are over 2,000 Mendelian diseases caused by known DNA variants, many patients who are suspected or have been clinically demonstrated to have rare genetic disorders do not receive a molecular diagnosis, often due to the genetic heterogeneity and to the relative inefficiency of the current sequencing technology.

Clinical Exome Sequencing (or Targeted Exome Capture) is a test intended for use in conjunction with the clinical presentation and other markers of disease progression for the management of patients with rare genetic disorders.

Conventionally diagnostic tests are conducted on a certain number of exons or genes after evaluation of the patient's clinical presentation. These tests are performed mainly for mendelian disorders for which it is well-established causal relationship between gene and disease. Instead NGS-based tests are performed mainly for patients without a clear clinical diagnosis or for patients who have negative test results for genes known to be associated with the disorder. Even if a large number of genes and genomic regions are investigated through WES or NGS-based comprehensive gene panel (or in some cases with whole genome sequencing), only a small fraction of these potentially could be associated with the patient's condition (Yu et al., 2012).

In a typical exome analysis, the starting number of variants is between 20.000 and 50.000. These variants have to be filtered to reduce false-positive calls by application of quality criteria or quality parameters (such as the total number of independent reads showing the variant and the percentage of reads showing the variant). The next step consists in removing or filtering out the variants outside the coding regions. This reduces the number of potential disease-causing variants approximately to 5000. Finally, the exclusion of known variants (consulting public database, literature, etc.) reduces the number of potential candidate mutations between 150 and 500 private non-synonymous or splice site that are prioritized as potential pathogenic variants (Gilissen et al., 2012).

WES can be applied for the analysis of several types of diseases (Rabbani et al., 2014):

- Monogenic: nearly two-thirds of these the molecular basis has not been discovered, even if over 6000 presumably monogenic disorders have been described;
- Heterogeneous monogenic: such as hearing loss, intellectual disabilities, autism spectrum disorders and *retinitis pigmentosa*;
- Common diseases and complex disorders: cardiovascular diseases, hypertension, obesity and diabetes;

- Cancer: in this field WES application is focussed on somatic mutation detection, driver mutation detection, mutation network reconstruction and identification of predisposing variants.

3.1 Clinical exome in mendelian disorders

- *Recessive*

The traditional approach used to identify genetic loci transmitted in association with the pathological phenotype was the linkage or segregation analysis which was directed to identify the regions shared between affected individuals in families with a large number of members and the final goal of gene discovery. Today it is possible to conduct a direct interrogation of “exome” for homozygous variants. Identification of recessive variants is very straightforward if the proband is a product of a consanguineous marriage. One of the first work of the application of WES technology identified recessive mutations in *WDR62* in Turkish consanguineous patients with severe developmental brain defects (Bilgüvaret et al., 2010).

Notably, variant calling and gene annotation of WES data of each individual exome contains about 10.000 non synonymous variants (depending on ethnicity and calling methods). A normal individual has been estimated to have 50–100 mutations in the heterozygous state that can cause a recessive Mendelian disorder when being homozygous (Rabbani et al., 2014).

More recently a large cohort of patients of similar genetic status were analysed using homo-mapping from the WES data. The authors uncovered 22 genes not previously identified as disease causing (Dixon-Salazar et al., 2012; Goh and Choi, 2012).

- *Dominant*

Heterozygous variants are generally more difficult to detect and analyse, not only due to their wide number as compared to homozygous variants but also because they are subjected to higher false positive and false negative errors. If large families are available with disease of interest (familial cohort approach) WES should be performed in more (ideally 8-12) family members. A recent example of such an approach is a WES study on familial amyotrophic lateral sclerosis (FALS), (Wu et al., 2012; Goh and Choi, 2012).

When large families with the disease are not readily available (not-familial cohort approach), WES could be performed on a number of unrelated patients with similar clinical manifestations and select genes that are commonly mutated in the patient cohort. In such approach rare functional variants, that could be present in longest genes, should be normalized using data from

population as a control. In a WES study of pseudohypoaldosteronism type II (PHAII), the variant burden of every gene from 11 unrelated patients was compared against that of 699 controls (Boyden et al., 2012).

Another approach based on *de novo* variant discovering should be performed if the disease phenotype is severe such that affects the reproductive fitness. Through the study of trios (unaffected parents and the proband) the variations that present only in the offspring and not in the parents, will be called a *de novo* variants. When there are unaffected siblings recruited simultaneously their *de novo* variations can be compared against those of the proband. Recent studies on trios and quartets have shown that rare *de novo* variants are associated with the risk of autism and *de novo* in multiple different genes may be involved in the etiology that underlies this disease (Neale et al., 2012; O'Roak et al., 2012; Sanders et al., 2012)

3.2 Clinical Exome in Common complex diseases

The common complex diseases are determined by the influence of genetic and environmental factors, and they are characterized by high genetic heterogeneity. For this reason the identification of disease-causing genes is very difficult and technical challenging. This includes conditions such as intellectual disability, autism, cardiac disease, hearing loss and diabetes. Traditionally linkage analysis was used in mutation discovery of family with extreme phenotype. However these mutations do not recapitulate the pathophysiology of disease for patients in the general population.

Great attention in complex disease studies was paid to profiling rare variants with allele frequencies of less than 1%, because large genome-wide association studies of common variants were not robust and did not explain very well the disease risk (Gibson et al., 2011). Several large-scale projects have been employed using WES technology, as for example the project launched by National Heart, Lung, and Blood Institute (NHLBI) to discover novel genes underlying cardiovascular disorders. In this project the exomes of 2,440 individuals were sequenced reporting many rare functional variants. In conclusion large numbers of subjects are necessary to discover variants significantly associated with the disease (Tennessen et al., 2012).

3.3 Clinical exome in diagnostic and gene discovery

More recently, NGS has been rapidly moving into the clinical diagnostics field and many NGS-based tests for multigene panels are available.

The first report that pointed out attention on correct diagnosis established by WES, was published by Choi et al. in 2009. They performed WES on a patient referred to Bartter syndrome, a rare disorder characterized by hypokalemia, in which it was discovered a novel homozygous mutation in SLC26A3 gene (gene known as causing congenital chloride-losing diarrhea (Choi et al., 2009). Disorders with phenotypic/genetic heterogeneity (e.g. neuromuscular diseases, cardiomyopathies, intellectual disability, cerebellar ataxias, retinal dystrophies) or patients with overlapping symptoms are difficult to be diagnosed and patients remain without a diagnosis.

Exome sequencing was used to interrogate a cohort of 118 probands with recessive neurodevelopmental disorders and identified disease-causing mutations in 19% of the probands. (Dixon-Salazar et al., 2012).

In 2013 Yang et al. was able to diagnose 25% of 250 of the previously undiagnosed patients as 80% of the patients were children with neurologic symptoms. In all, scientists identified 86 mutated genes that are known to cause genetic diseases in 62 of the 250 patients. Among the 62 patients who received a diagnosis, 33 had autosomal dominant disease, 16 had autosomal recessive disease, and 9 had X-linked disorders (Yang et al., 2013).

Zhang et al., identified a novel deletion mutation in Emerin gene D which results in almost a complete loss of emerin protein in a large Chinese family (Zhang et al., 2014).

NGS is used to identify mutations in 321 exons representing 12 genes involved with congenital muscular dystrophy (Valencia et al., 2012).

In a recent work were investigated 405 unresolved families with X-linked intellectual disability and the researchers found that 20% of the families contained pathologic variant. In 19 families detected likely causative protein truncating and missense variants in 7 novel genes (CLCN4, CNKSR2, FRMPD4, KLHL15, LAS1L, RLIM and USP27X) and potentially deleterious variants in 2 novel candidate XLID genes (CDK16 and TAF1), (Hu et al., 2015).

Another group analysed by WES 110 index patients with Inherited peripheral neuropathies (IPN) were undetermined after a screening for mutations in common genes. They identified 41 missense variations in the known IPN genes. Nine variants (8%) were previously reported and considered to be pathogenic in these families, twenty (11%) were novel variants, and the remaining twenty variants were confirmed as polymorphisms (Drew et al., 2015).

WES has a useful application in disease treatment (e.g. Crohn disease diagnosed to a 15-month-old infant with immune deficiency has been facilitated the correct management of the patient), screening (e.g. neonatal screening of *diabete mellitus*) and prenatal diagnosis using fetal DNA in maternal serum to discover aneuploidies.

3.4 Strengths and weaknesses of clinical exome

First strength of WES is represented by its costs; at mean coverage depth of 100× still costs five times less than whole genome sequencing (WGS) at mean coverage depth 30×.

The second one is the amount of data per patient is approximately a sixth of WGS resulting in reducing processing time and less of a burden in terms of data storage. The third is the possibility to analyse a large number of patients in a parallel and robust fashion.

However, it is important to understand the limitations of WES technology. First, some protein-coding regions might not be covered due to an incomplete annotation of the human genome. Second, WES does not cover non-coding elements, including non-translated regions, enhancers, and long-noncoding RNA potentially functional. Third, WES is characterized by a limited ability to detect structural variations, such as copy-number variations.

Finally, another limitation is the occurrence of false positive (false variant that is called true) due to an incorrect alignment with the reference sequence, systematic sequencing errors, technical limitation of the machine, and false negative (true variant that is called failed) primary due to low coverage, low enrichment in the region of interest, alignment with repetitive elements of the genome.

Currently, from the exome sequencing data it is possible to achieve ~98% sensitivity and 99.8% specificity, however, it is technically challenged to detect and to interpret rare heterozygous variants because heterozygous variants calling is more susceptible to the technical errors and requires higher read depth (Goh and Choi, 2012).

4. Neuromuscular diseases

Neuromuscular diseases (NMDs) is a wide term covering heterogeneous rare disorders caused by defects in muscles (various types of dystrophies, myopathies, and ion channel diseases), motor neurons (amyotrophic lateral sclerosis, spinal muscular atrophies), neuromuscular junction (myasthenic syndromes), nerves (neuropathies) and cerebellum (hereditary ataxia). It has

estimated that between 6 to 8 million people worldwide are affected by NMDs (Vasli et al., 2010; McDonald et al., 2012; Scotton et al., 2014).

Over 80 different genetically defined types of muscular dystrophies are discovered and categorized into different subgroups based on the age of onset, the specific muscles involved, and common clinical features (Ankala et al., 2014). Signs and symptoms of various NMDs may be very different. For example while the anterior horn cells are affected in spinal muscular atrophy (SMA, OMIM 253300), peripheral nerve defects cause the Charcot Marie Tooth (CMT, OMIM 118200) neuropathy (Scotton et al., 2014).

The major clinical characteristics of the disease group include muscle degeneration and wasting, progressive muscle weakness, hypotonia, and although at very variable levels, elevated serum creatine kinase levels. Cardiac involvement might also be present, accounting for higher morbidity and mortality.

NMDs are characterized by:

- High genetically heterogeneity: more than 300 genes are implicated on disease's pathogenesis;
- Mutations occurring in largest genes: these genes sometimes are not fully tested in diagnostic but have been analysed at first for mutation in hot spot regions;
- Clinical heterogeneity: overlap of symptoms in different NMDs diseases and the same mutation can cause different clinical phenotypes with different severities;
- Unidentified genes: about 40 % of patients with NMDs do not have a genetic diagnosis.

4.1 Congenital muscular dystrophies

Congenital muscular dystrophies (CMDs) and limb-girdle muscular dystrophies (LGMDs) are the two major subgroups of NMDs and are characterized by hypotonia and weakness, variable appearance of contractures and dystrophic changes on skeletal muscle biopsy.

The congenital muscular dystrophies present either congenitally or during the first 6 months of life, while LGMDs present in late childhood, adolescence or adulthood (Cirak et al., 2013; Voit, 1998; Bonnemann and Finkel, 2002).

The typical presentation observed in CMDs is a “floppy baby”; however, in patients with milder symptoms, axial muscles of the spine are more involved with pronounced head lag, as it happens often in selenoprotein 1 (SEPN1)-and lamin A/C (LMNA)-related CMDs. Some symptoms such as marked facial weakness are unlikely in CMD (generally occur in patient with congenital myopathies). Cardiomyopathy can develop in the second decade of life, due to FKTN-, fukutin-

related protein (FKRP), and protein-O-mannosyltransferase 1 (POMT1)-related CMDs/limb-girdle muscular dystrophy (LGMD) phenotypes and the occurrence at birth is rather unlikely. Sudden cardiac death has been reported almost exclusively in LMNA-related CMD. Brain involvement can simply manifest as mental retardation (MR) without MRI structural abnormalities (Bertini et al., 2011).

Diagnosis of CMDs is based on clinical findings, brain and muscle MRI, muscle biopsy histology and immunohistochemistry, skin biopsy and cultured immunohistochemical staining, as well as molecular genetic testing.

Information on prevalence and incidence of CMDs is insufficient because of the lack of diagnostic genetic in the past 10 years. Currently, the achievement of a molecular diagnosis is important for phenotype-genotype correlations, genetic and prenatal counseling, prognosis and management, and in particular concerning the availability of clinical trials and treatments.

A major pathological subgroup of CMDs and LGMD is characterized by the reduction in the functional glycosylation of aDG (aDGpathies) and the phenotypic spectrum include the Walker-Warburg syndrome (WWS; Online Mendelian Inheritance in Man [MIM*236670]; Walker, 1942; Warburg, 1971), FCMD [MIM*253800]; Fukuyama, 1960), muscle-eye-brain disease (MEB; [MIM*253280]; Raitta et al., 1978), congenital muscular dystrophy 1C (FKRP-related or MDC1C; [MIM*606612; Brockington et al., 2001a), and congenital muscular dystrophy 1D (LARGE-related or MDC1D; [MIM*608840]; Longman et al., 2003) as well as the allelic LGMD presentations for most of these genes (Cirak et al., 2013; Bertini et al., 2011; Muntoni and Voit, 2004).

An essential step during the glycosylation process is the unique O-mannosylation sugar moiety that is involved in the interaction of α -dystroglycan with the protein of the extracellular matrix such as laminin, perlecan, agrin, neurexin and pikachurin (Cirak et al., 2013; Waite et al., 2012; Kanagawa and Toda, 2006). The α -dystroglycan protein anchors the structural framework inside each cell (cytoskeleton) to the proteins and other molecules outside the cell (extracellular matrix). In skeletal muscles, glycosylated α -dystroglycan stabilizes and protect muscle fibres, and in the brain it directs the migration of neurons during early development (fig.6).

Mutations in eight genes (POMT1; [MIM*607423]; Beltran-Valero de Bernabe et al., 2002), POMT2; [MIM*607439]; von Reeuwijk et al., 2005; POMGNT1; [MIM*606822]; Yoshida et al., 2001; FKTN; [MIM*607440]; Kobayashi et al., 1998; FKRP; [MIM*606596]; Brockington et al., 2001a; LARGE; [MIM*603590]; Longman et al., 2003; DPM3; [MIM*605951]; Lefeber et al., 2009; DOLK; [MIM*610746]; Lefeber et al., 2011) coding for glycosyltransferases or other proteins involved in α -dystroglycan glycosylation pathway are responsible for 50–60% of

the cases classified as dystroglycanopathy (Godfrey et al., 2007; Mercuri et al., 2009; Messina et al., 2010; Devismeet al., 2012; Cirak et al., 2013).

Cirak and colleagues mapped a novel disease locus to chromosome 7p21 in a family with loss of α -dystroglycan glycosylation (Cirak et al., 2009) and recently Willer and colleagues discovered that bi-allelic loss-of-function mutations in the isoprenoid synthase domain containing (*ISPD*) gene, which maps to chromosome 7p21, are a frequent cause of Walker–Warburg syndrome (Willer et al., 2012). During 2013, the working group of Cirak published another document where they reported the identification of nine patients from seven families who carried mutations in *ISPD*, with phenotypes ranging from congenital muscular dystrophy to LGMD. These families included the original family mapped to 7p21 (Cirak et al., 2013). Furthermore, a new homozygous *ISPD* gene mutation was discovered in two cousins from consanguineous parents family of Pakistani origin (Baranello et al., 2015).

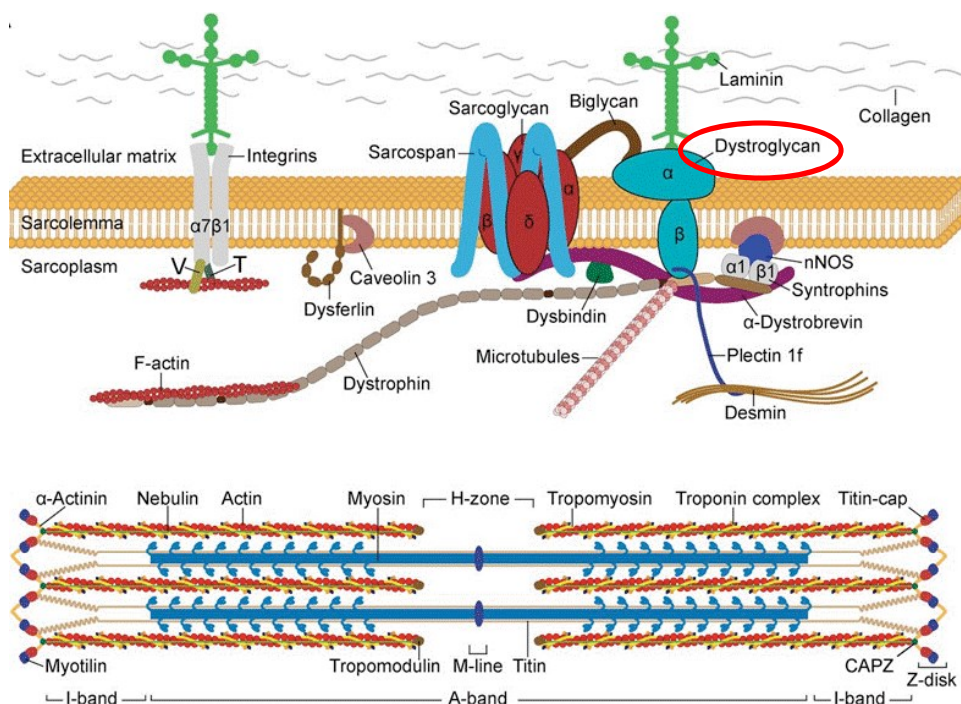


Figure 6. Sarcolemmal proteins and sarcomere structure. Dystrophin-associated protein complex (DAPC). Dystroglycan is indicated by red circle. DAPC is a multimeric protein complex that connects the intracellular cytoskeleton of a myofiber to the extra cellular matrix, which is composed of laminin, collagen, and other proteins. The glycosylated α -dystroglycan directly interacts on one side with $\alpha 2$ subunit of muscle specific laminin and with the transmembrane β -dystroglycan on the other side. Also β -dystroglycan binds the cysteine-rich domain of dystrophin which interact with microtubules. At the bottom is the basic contractile unit of skeletal muscle (adapted from Rahimov and Kunkel, 2013)

4.2 Congenital Myopathies

Congenital Myopathies are a group of muscle disorders characterized clinically by hypotonia and weakness, generally from birth, and a static or slowly progressive clinical course. Classically, the congenital myopathies have been classified on the basis of the major morphological features seen on muscle biopsy: rods (in nemaline myopathy), cores (in central core disease and multiminicore disease), central nuclei (in centronuclear/myotubular myopathy) and selective hypotrophy of type I fibres (in congenital fibre type disproportion), (Dubovitz et al., 1969; Cardamone et al., 2008).

The relationship between each congenital myopathy (defined on histological grounds), and the genetic cause are complex because of the genetic heterogeneity; in fact mutations in the same gene can cause different muscle pathologies, and the same genetic mutation can lead to different pathological features in members of the same family or in the same individual at different ages.

Although there is present a clinical overlap between congenital myopathies and other neuromuscular disorders (e.g. congenital muscular dystrophy), common clinical findings are: the presence of prominent facial weakness with or without ptosis, generalized hypotonic ('frog-leg') posture with hyporeflexia, and weakness and dysfunction of the respiratory and bulbar muscles. Intelligence is usually normal (North et al., 2014).

In neonates, weakness is present as profound generalized; in other patients it is manifested during childhood with delayed motor milestones, or even later in life with symptoms of proximal weakness. Weakness is generalized or prominent in limb-girdle and proximal limb muscles.

Congenital myopathies arise in childhood with hypotonia, feeding and respiratory difficulties, and delayed motor milestones. Milder cases may present contracture, scoliosis, and respiratory insufficiency later in life. Typical signs include proximal or generalized weakness, hypotonia and hyporeflexia, reduced muscle bulk, and dysmorphic features secondary to muscle weakness (*pectus carinatum*, scoliosis, a high arched palate, and elongate *facies*). Weakness is usually static or slowly progressive. Cardiac involvement is rare. Muscle biopsy shows changes specific to the myopathy, without necrotic or fibrotic changes.

To date, eight genes have been identified for nemaline myopathy. In particular mutations in the nebulin gene (*NEB*) recapitulate for 40–50% of nemaline myopathy cases, although the exact proportion has yet to be definitively proven. All patients with *NEB* mutations to date have autosomal recessive disease. Most patients (~ 90%) affected by central core disease (CCD) have dominant changes in the ryanodine receptor gene (*RYR1*). Around 60% of *RYR1* CCD mutations are in the CCD hotspots including the C-terminal region (North et al., 2014). Patients with

classic CCD typically present in infancy or early childhood with mild to moderate hypotonia, motor-development delay, proximal muscle weakness, and occasionally with congenital hip dislocation. A subgroup of patients with *RYR1*-associated myopathy have a more severe or even lethal neonatal form of the disorder, with features such as decreased fetal movement, polyhydramnios, arthrogryposis, kyphoscoliosis, or respiratory distress (Bharucha-Goebel et al., 2013). Moreover *RYR1* is the most common cause of true core-rod myopathy, and have been described both dominant and recessive mutations. In the multimimicoid disease the first gene that should be investigated is selenoprotein 1 (*SEPN1*) and *RYR1* as the second most probably causing gene. Slow α -tropomyosin (*TPM3*) is the most common known genetic cause of congenital fibre type disproportion (*CFTD*), accounting for 25–40% of patients and usually follows autosomal dominant inheritance. Many mutations are de novo dominant mutations. Ryanodine receptor (*RYR1*) mutations have been found in around 20% of patients and may be associated with marked fibre size disproportion (>50%). All patients up to date had autosomal recessive disease. Mutations in the myotubularin gene (*MTM1*) have been identified in severely affected patients of centronuclear myopathies, and also ryanodine receptor (*RYR1*) mutations usually cause autosomal recessive CNM (North et al., 2014).

Ryanodine receptors (RyRs) represent the sentinels of massive intracellular calcium stores contained within the sarcoplasmic reticulum (Capes et al., 2011), and in response to the sarcolemma depolarization of T-tubules, RyRs mediates the release of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm and thereby plays a key role in facilitating mobilization of the myofilaments and triggering muscle contraction (fig.7).

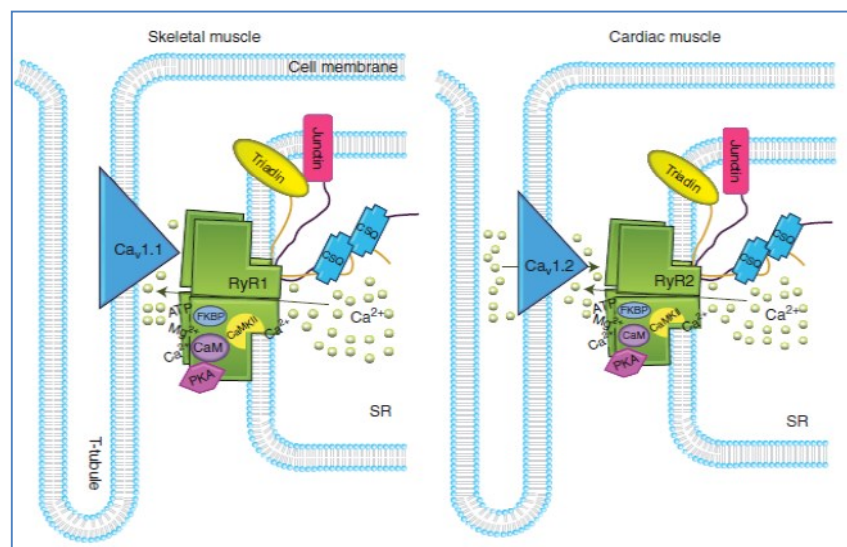


Figure 7. Schematic diagram of RyRs functions in excitation-contraction coupling. Depolarization of plasma membrane can lead to opening of voltage-gated calcium channels (CaV) which conducts Ca^{2+} influx into the cytoplasm. RyRs in SR membrane sense this initial signal and amplify it releasing more Ca^{2+} from SR store (process known as Ca^{2+} -induced Ca^{2+} release, CICR). This will provides the bulk of the Ca^{2+} required for contraction. Many cytosolic metabolites and proteins participate to release-sequestration process: protein kinase A (PKA), FK506 binding protein (FKBP12), calmodulin (CaM), Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), calsequestrin (CSQ), triadin, junction (Lanner et al., 2010)

4.3 The distal hereditary motor neuropathies

The distal hereditary motor neuropathies (dHMNs) are heterogeneous group of conditions, both phenotypically and genetically, prevalently affecting motor neurons in the peripheral nervous system. The affected individuals present progressive weakness and wasting beginning in the distal muscle of the limbs and have no notable sensory symptoms (Kennerson et al., 2010). Many forms of dHMN have minor sensory abnormalities, and it is possible to overlap between the axonal forms of Charcot Marie Tooth (CMT2) and dHMN (Rossor et al., 2012).

The classification of disease is based on a system proposed by Harding in 1993 (Harding, 1993), in which seven categories are defined: I, II, V and VII autosomal dominant, and types III, IV, VI autosomal recessive.

Types I and II are typical dHMNs because the early effect is in the lower limbs and is present with distal wasting and weakness in either childhood or adulthood respectively. These phenotypic categories are genetically heterogeneous; in fact mutations in two genes, *HSBP1* or *HSBP8*, are implicated. DHMN with pyramidal signs can be caused by mutations in *BSCL2* and *SETX*. Type V is characterized by upper-limb onset and can be due to mutations in *BSCL2* or *GARS*. Type VII is defined by vocal-cord paralysis and the gene involved are *DCTN1*, *TRPV4* or unknown gene. Type III and IV are chronic forms of dHMN and the causing gene is unknown. Type VI occurs in infancy and display a phenotype characterized by distal weakness and respiratory insufficiency and it is due to mutations in *IGHMBP2*. However more than 80% of patients with dHMN have mutations in unknown genes (Dierick et al., 2008; Rossor et al., 2012). The causative genes encode for proteins with diverse functions: protein misfolding, RNA metabolism, axonal transport, and cation-channel dysfunction (Rossor et al., 2012).

Recently Kennerson et al. (Kennerson et al., 2010) mapped an X-linked form of distal motor neuropathy to chromosome X in two large unrelated families. The region of genetic linkage included *ATP7A*, which encodes a copper-transporting P-type ATPase mutated in patients affected by Menkes disease, a severe infantile-onset neurodegenerative and its milder variant, occipital horn syndrome (OHS). The affected individuals presented an axonal distal motor neuropathy in the lower limbs with mild sensory involvement and the age of onset was between 2 and 61 years. Serum copper levels were within the normal range (Kennerson et al., 2010; Rossor et al., 2014).

ATP7A and *ATP7B* are responsible for transferring copper from the cytosol into Golgi lumen for incorporation into copper-dependent enzymes (fig.8).

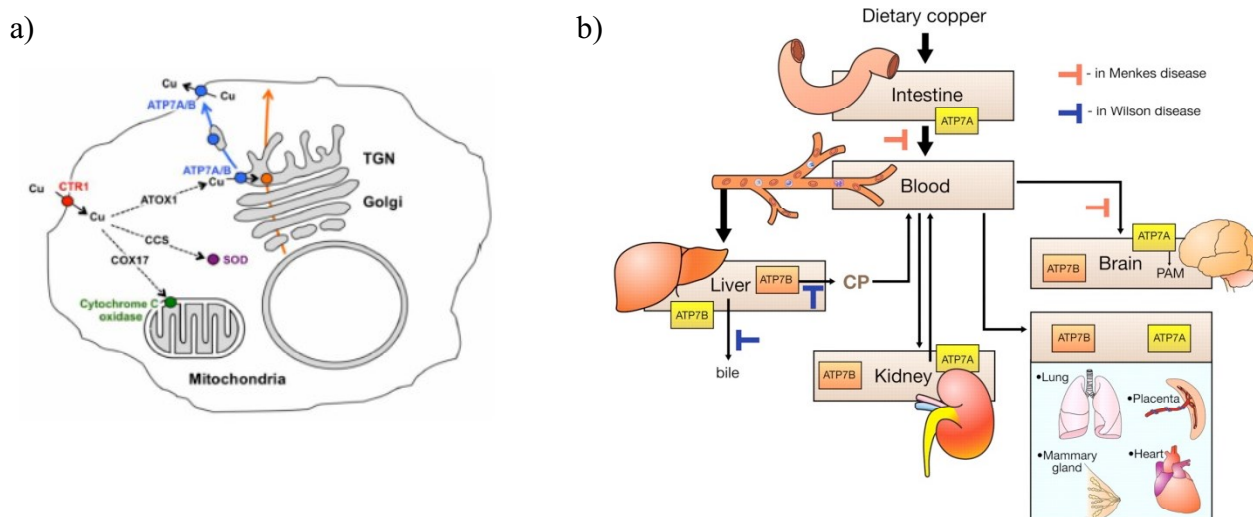


Figure 8. a) Intracellular pathways of copper distribution. Cu^{2+} is absorbed through the apical CTR1 channel by the enterocytes in the small intestine and by hepatocyte in the liver and it transferred to chaperones (such as ATOX1) which ferry it to ATP7A/B in the Golgi and to intracellular cuproenzymes: Cu–Zn superoxide dismutase (in the cytosol), cytochrome c oxidase (in the mitochondria). In the Golgi ATP7A/B load Cu on the newly synthesized cuproenzymes which are involved in the biosynthetic pathways (orange arrow). When intracellular Cu^{2+} increases, ATP7A and ATP7B moves towards post-Golgi compartments and plasma membrane, where they drives the excess of Cu^{2+} from the cell (blue arrow). In the enterocyte this process happen across the basolateral surface into the portal circulation. **b) Copper distribution in the body and diseases associated with ATP7A/B deficiency.** In Menkes disease, *ATP7A* is inactivated and copper export from the enterocytes is greatly impaired. As a result, copper accumulation in intestinal cells causes less copper delivery to the blood, resulting in reduced metal supply to other tissues. In Wilson disease, mutations in *ATP7B* gene impairs its activity and trafficking, blocking Cu delivery to ceruloplasmin (CP) and its release into the bile, causing a toxic Cu^{2+} accumulation in the liver (adapted from Lutsenko et al., 2007; Polishchuck and Lutsenko, 2013).

This metal is involved in many biochemical processes such as respiration, neurotransmitter synthesis, activation of neuropeptides and hormones, protection from oxidative damage, myelination, pigmentation, and iron metabolism (Lutsenko, 2010; Nevitt et al., 2012). Although it is an essential metal, its oxidation produced reactive oxygen species and unchelated Cu^{2+} can be toxic to cells. Therefore, within the cells a complex network of regulatory mechanisms is developed to control Cu^{2+} levels both at the cellular and systemic levels and to satisfy its metabolic request. Copper is taken into the cell by high affinity copper transporter on the plasma membrane and inside the cell it is bound by chaperone proteins (such ATOX1) through direct interaction (Lutsenko et al., 2007; Lutsenko 2010; Nevitt et al., 2012) with N-terminal end that contains six-copper binding domain. When intracellular copper concentration increases, ATP7A moves from trans Golgi network (TGN) and reaches the plasma membrane to remove the copper in excess. After this process, when the basal copper concentration is $\approx 0.5\mu\text{M}$, ATP7A returns to the TGN. To catalyze the translocation of copper across cellular membranes they undergo ATP-

dependent cycles of phosphorylation and dephosphorylation to catalyse the translocation of Cu^{2+} across cellular membranes (fig.8), (Telianidis et al., 2013).

Mutations in *ATP7A/B* or in the proteins that govern their trafficking (exit from Golgi and their retrieval) disrupts, in turn, the homeostatic balance, resulting in copper deficiency (Menkes disease) or copper overload (Wilson disease), (Polishchuk and Lutsenko, 2013).

5. NEUROMICS project: a challenge to rare diseases

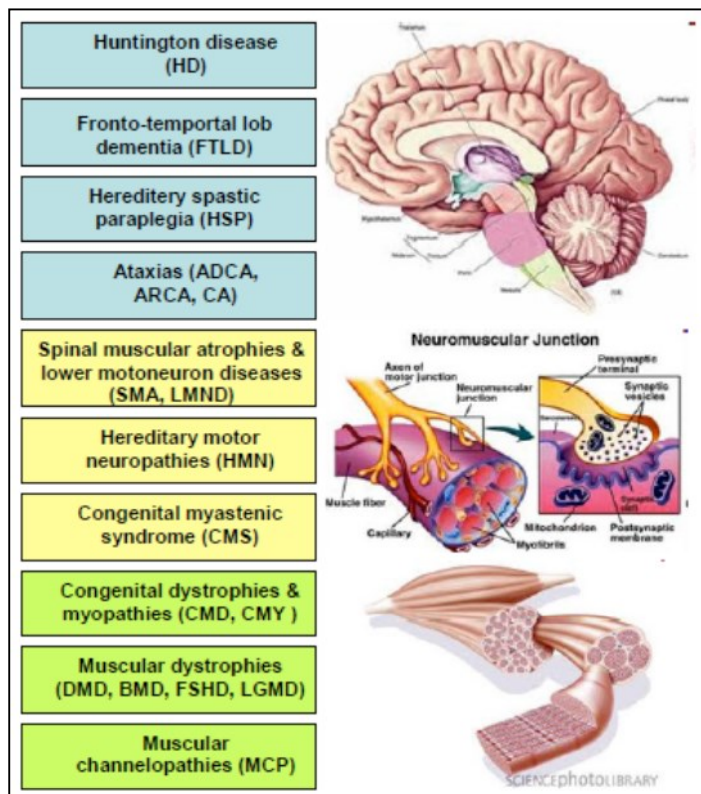


Figure 9. Diseases and disease groups in the focus of NEUROMICS research

Our study was performed within the Neuromics project. In fact an *Integrated European – omics research project – for diagnosis and therapy in rare neuromuscular and neurodegenerative diseases* started on October 1st 2012. The project will go on until 2017 with the aim of increasing the benefit to patients and their carers and clinicians for at least 10 major NDD and NMD (see fig. 9) using cutting-edge Omics technologies. The consortium consists of 14 European academic groups, 4 associated overseas partners, 1 big company and 4 SMEs and has top-level expertise on more than 500 genetically defined diseases comprising more than 600.000 patients in Europe. It is

estimated that about 500.000-600.000 patients are suffering from rare NDD/NMD in Europe (table 2). UNIFE (Alessandra Ferlini) is a full partner of the project.

NEUROMICS will focus on fronto-temporal dementia (FTLD) with or without ALS, Huntington disease (HD), spinocerebellar ataxias (SCA), hereditary spastic paraplegias (HSP), spinal muscular atrophies (SMAs), SMA with arthrogyriposis and lower motor neuron diseases (LMND), hereditary motor neuropathies (HMN), congenital myasthenic syndromes (CMS), congenital muscular dystrophies and congenital myopathies (CMD and CMY), muscular dystrophies [Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), limb

girdle muscular dystrophy (LGMD), fascio-scapulo humeral dystrophy (FSHD)], and muscular channelopathies (MCP).

Common to all NDD/NMD covered in NEUROMICS is a large genetic heterogeneity with 10-50 known genes per disease group (except for HD and DMD/BMD). Still, between 30-80% of patients remain undiagnosed: e.g. 50% of autosomal inherited ataxias, HSPs, and CMDs or up to 80% of recessive inherited ataxias, distal SMAs, SMA with arthrogryposis

Disease	Prevalence	Patients in Europe
FTLD	3-10 per 100.000	15.000-50.000
HD	3 - 7 in 100.000	35.000
Ataxias	20 in 100.000	200.000
HSP	2-10 in 100.000	10.000-50.000
SMA/LMND	5-10 in 100.000	20.000-40.000
HMN	1 in 2.500	200.000
CMS	1 - 10 in 1 Mio	500 – 5.000
CMD	7 - 12 in 100.000	40.000
D(B)MD	1 in 20.000	25.000
Dysferlinopathies	1 in 50.000	10.000
FKRP	0.5 in 100.000	2.000
MCP	0.5 to 1 in 40.000	6.000-12.000

Table 2. Estimates of prevalence of NDDs/NMDs in Europe (patient numbers are estimated from numbers of single countries and extrapolated to the EU, or are calculated from heterozygote frequencies)

or HMNs. Furthermore, almost all sporadic cases - which include a large NDD/NMD cohort - are still undiagnosed. Thus, it is a major challenge to identify the genetic cause for more than half of the currently unknown causes for NDD/NMD (~50% of the patient population).

Neuromics will focus on the following aims:

1. screening more than 1100 patients with unknown genetic cause by **Whole Exome Sequencing** (WES) using next-generation technologies (NGS), thereby increasing the number of known disease genes for the most heterogeneous diseases from approximately 50% to 80%;
2. increasing patient cohorts by **large-scale genotyping** using 3 gene panel enrichments for overlapping disease groups (NDD, NMD, SMA/LMND) combined with NGS of so far unclassified patients and subsequent phenotyping;
3. developing **biomarkers** with a focus on pre-symptomatic implementation, for application in diagnosis and clinical trials;
4. combine Omics approaches to better understand **pathophysiology** guiding therapeutic approaches;
5. identifying **disease modifiers** through the characterization of cohorts with extreme early and late age of onset (SMA, HD, CMS, SCA);
6. developing **target-driven therapies** for muscular dystrophies such as DMD, LGMD2B, CMD, other NMD, and polyQ expansion diseases, subsequently to be translated to other disease groups using the expertise of the consortium;

7. improving **infrastructure and ontologies** towards their application for NDD/NMD and to support translational research and networking with Support IRDiRC and RD-Connect project.

AIM

Aim of my work was to identify disease causing genes involved in neuromuscular diseases and performing validation analysis. Among the several families we studied within the Neuromics project, I focused my attention on three “family of four”, which were analysed by whole exome sequencing (WES) in two families for the diagnosis of congenital muscular dystrophy and congenital myopathy, and multi-gene panel test on the third family with distal axonal motor neuropathy. The next generation sequencing has become an effective strategy to study clinically and genetically heterogeneous diseases, such as neuromuscular disorders, and can accelerate the patient’s access to a better healthcare and disease management.

MATERIALS AND METHODS

1. Sample Selection

We selected three Italian “family of four”: family 1 with one female affected by congenital muscular dystrophy, family 2 with two siblings (male and female) affected by congenital myopathy, and family 3 with two male brothers affected by distal axonal motor neuropathy and autonomic dysfunction.

2. DNA Extraction

DNA was extracted in all samples from whole blood by nucleon BACC3 kit (GE Healthcare Life Sciences, UK) to obtain a DNA with high molecular weight, not degraded and with 260/280 ratio about 1.8 and 260/230 ratio about 2.0. The quality of DNA was assessed by Nanodrop ND-1000 instrument (NanoDrop Technologies, Wilmington, DE).

3. Sequencing Analysis

- *Family 1-2:*

WES analysis was performed in collaboration with deCODE genetics company. Briefly, the protocol provides that 5µg of DNA are fragmented by Covaris into fragments of 150-200 bp. The fragmented DNA are captured according to the protocol by SureSelect Human All Exon Kit (Agilent Technologies, Inc., Santa Clara, CA, USA). This kit is capable of capturing 38Mb of the human genome (about 1.22%), which corresponds to the entire coding region of the human genome reported in the NCBI database. The sequencing of the library is performed by Illumina Genome Analyzer IIe, using the 2x51 protocol.

- *Family 3:*

Gene Panel analysis was performed in collaboration with deCODE genetics company. The analysis was carried out on Ion-Torrent Personal Genome Machine using a custom LMND gene panel. The panel includes 21 genes associated to ALS, 13 genes associated to SMA, 16 genes associated to HMN and 14 genes associated to CMT. The analyzed fragments cover the coding regions as well as 5'- and 3'-UTRs of 64 selected neuromuscular disease genes (table 1), that

were enriched from 10 ng genomic DNA using the Ion AmpliSeq Enrichment kits. Sequencing was performed bidirectionally as single-end sequencing achieving a mean coverage of >300fold. Variants outside the covered regions/genes are not detected. Variants in regions, that are insufficiently covered (coverage below 20fold) or within highly homopolimeric regions, might also escape detection. Small insertion and deletions, larger structural aberrations and epigenetic mutations are not (reliably) detected.

AARS	alanyl-tRNA synthetase
ALS2	Asin 2
ANG	Angiogenin
AR	Androgen receptor
ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1
ATP7A	ATPase, Cu ⁺⁺ transporting, alpha polypeptide
ATXN2	Ataxin 2
BICD2	Bicaudal D homolog 2 (Drosophila)
BSCL2	Berardinelli-Seip congenital lipodystrophy 2 (seipin)
CCDC78	coiled-coil domain containing 78
CHMP2B	Cromatin modif. protein 2B / charged multivesicular body protein 2B
DAO	D-amino acid oxidase
DCTN1	dynactin 1
DCTN2	Dynactin 2 (p50)
DNAJB2	HSJ1 chaperone
DNM2	dynammin 2
DYNC1H1	Dynein, cytoplasmic 1, heavy chain 1
FBLN5	Fibulin 5
FBXO38	F-box only protein 38
FIG4	SAC domain-containing inositol phosphatase 3
FUS	Fusion, derived from t(12;16), malignant liposarcoma (FUS; FUS/TLS)
GARS	glycyl-tRNA synthetase
GDAP1	Ganglioside-induced differentiation-associated protein 1
HINT1	Histidine triad nucleotide-binding protein 1
HSPB1=27	heat shock 27kDa protein 1
HSPB3=HSPL27	heat shock 27kDa protein 3
HSPB8=22	heat shock 22kDa protein 8
IGHMBP2	Immunoglobulin mu binding protein 2
KIF1A	kinesin family member 1A
KIF1B	kinesin family member 1B
KIF1C	kinesin family member 1C
KLHL9	Kelch-like homologue 9
LMNA	lamin A/C
LRSAM1	Leucine-rich repeat- and sterile alpha motif-containing 1
LUM	Lumican
MED25	mediator complex subunit 25
MFN2	Mitofusin 2
MPZ	myelin protein-zero
MUSK	Muscle, skeletal, receptor tyrosine kinase
MYH14	Myosin heavy chain 14 (non-muscle)
MYH7	myosin, heavy polypeptide-7, cardiac muscle, beta gene
NEFH	Nurofilament heavy chain
NEFL	neurofilament, light polypeptide
OPTN	Optineurin
PFN1	Profilin-1
PLEKHG5	Pleckstrin homology domain containing, family G member 5
PRPH	Pripherin
REEP1	receptor accessory protein 1
SCML2	Sex comb on midleg, drosophila, homolog-like 2
SCO2	SCO cytochrome oxidase deficient homolog 2
SETX	Snataxin
SIGMAR1	Sigma nonopioid intracellular receptor 1 gene
SLC18A3	solute carrier family 18 (vesic. acetylchol.) member 3; VACHT
SLC5A7	solute carrier family 5 (choline transporter) member 7
SMN1	Survival of motor neuron 1
SOD1	Speroxid dismutase ^1
SPG11	Spatacsin
TAF15	TAF15 RNA Polymerase II
TARDBP	TAR DNA-binding protein = TDP-43
TFG	TRK-fused gene
TRPV4	transient receptor potential cation channel, subfamily V, member 4
UBA1	Ubiquitin-like modifier activating enzyme 1
UBQLN2	Uiquilin 2
VAPB	VAMP (vesicle-assoc-membrane protein)-assoc protein B and C
VCP	Vlosin-containing protein

Table 1. List of 64 genes of lower motor neuron diseases gene panel analyzed with Ion Torrent sequencing method. In the first column are reported the acronyms of each gene, and in the second column are reported the entire names

4. Data Analysis

The analysis of raw data was fully performed by deCODE genetics company, the genetic and statistical analysis, the mutation effect prediction, the validation and segregation analysis was performed by myself in collaboration with the Colleague in the Unit of Medical genetics.

The first step of the WES analysis consists in a quality control of the reads (FASTQ format) performed by FastQC [<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>] through which the low quality reads were discarded. On the contrary the high quality reads were aligned with the human reference genome using BWA (Li and Durbin, 2009).

The generated files in SAM format were then converted into a binary alignment format (BAM). The Genome Analysis Toolkit (GATK) software was applied to decipher variations including changes in a single polypeptide (SNP) and small insertions/duplications (DIP).

Finally, the identified variations were annotated by ANNOVAR (<http://www.openbioinformatics.org/annovar/>); dbSNPs (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) or 1000 genomes (<http://www.1000genomes.org/>) in silico tools were used to verify if a variation exist as polymorphism in the general population, and also to know its genomic location and the relative effect (sense, missense, nonsense, frameshift mutations). The pathogenic prediction of the nucleotide change was established by SIFT (<http://sift.jcvi.org/>) and PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) algorithms.

Analysis of the families 1 and 2 were performed applying candidate gene interrogation and autosomal recessive inheritance to find homozygous variations in affected patients and heterozygous in the parents. Prioritization of the variants was carried out by Clinical Sequence Miner Software provide by deCODE selecting the following additional filters:

- maxAf (allele frequency): 0.01%
- minHeteroCall: 30%
- minHomoCall: 85%
- quality filters (as quality of run)
- type of mutation: high impact (transcript ablation, splice donor variant, splice acceptor variant, stop gained, frameshift variant, stop lost, initiator codon variant) and moderate impact (in-frame insertion, in-frame deletion, missense variant, transcript amplification, splice region variant, incomplete terminal codon variant).

Identified variations were prioritized for the pathogenic prediction and the rare allele frequency under 1% of the population or even 0,5% for very rare allele.

Data analysis on the family 3 was carried out by TMAP and Ion plugins, SureCall V1.0 and SeqNext V4.1.2 bioinformatics tools. At the end was performed a comparison of results with

entries in public database such as Human Gene Mutation Database HGMD (www.hgmd.cf.ac.uk) and Exome Variant Server (evs.gs-washington.edu/) that contains exome data from more than 6500 healthy control probands. Potentially pathogenic variants were tested *in silico* using Human Mutation Taster (www.mutationtaster.org) and Polyphen2 (genetics.bwh.harvard.edu/pph2/) algorithms.

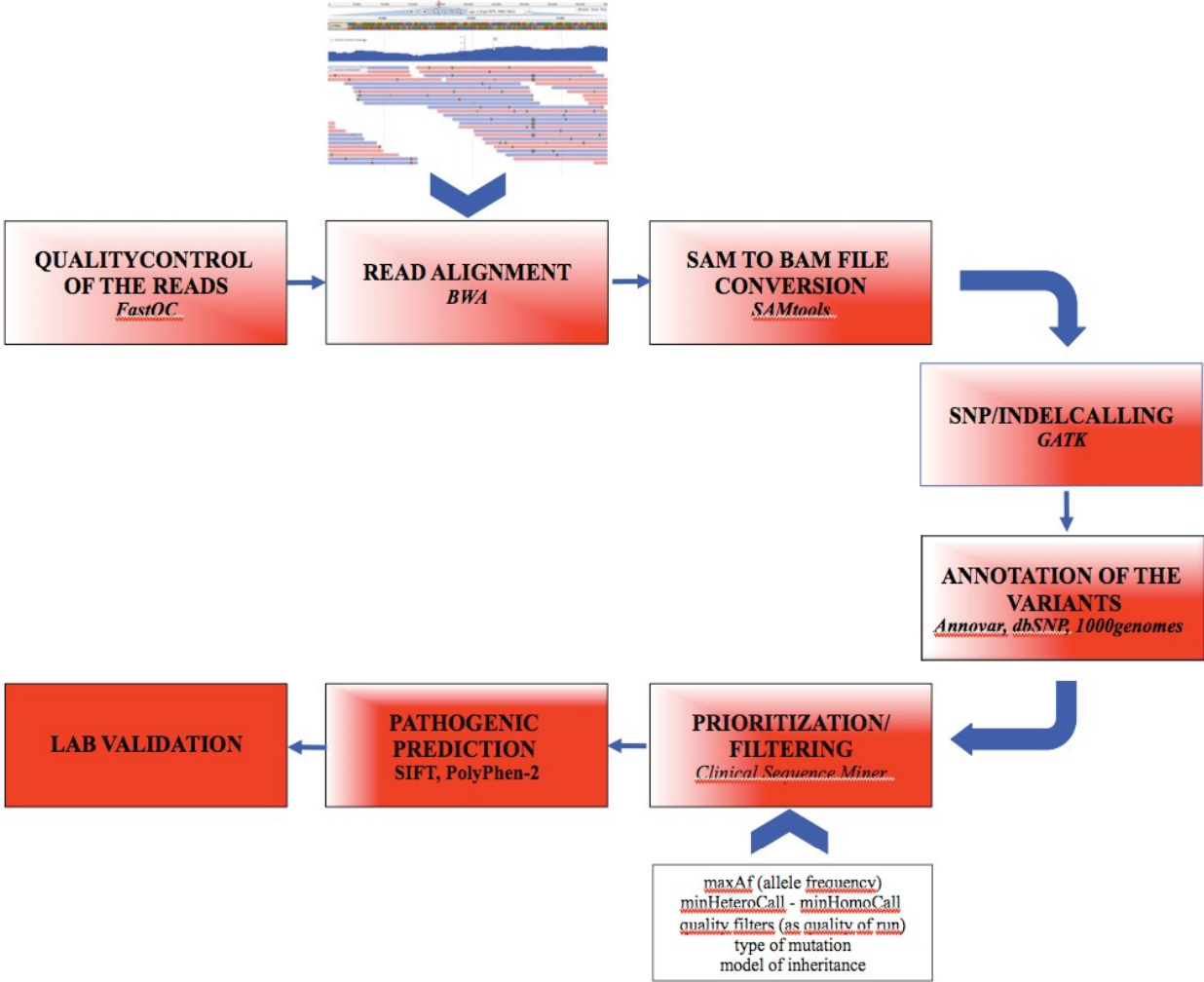


Figura 1. Whole exome sequencing data analysis workflow

5. Candidate Gene Sequencing

Sanger sequencing was conducted on ABI 3130XL Genetic Analyzer (Applied Biosystems) using BigDye_ v.3.1 Cycle Sequencing Kits (Applied Biosystems).

We performed primer design by bioinformatics tool Primer3 (<http://primer3.ut.ee/>). The primers sequences are reporting in the following table:

Gene Name	Exon	Sequence
RYR1	103/104	Forward 5'-GGGGTGAGATTAGGGAAATGGG-3'
		Reverse 5'- CTTCTGATTGGCTGGCAGGG-3'
ATP4A	6	Forward 5'- GGCCCCTGTCCCTCATCTG-3'
		Reverse 5'-GGAGAGGGTAGTGACAGTGGG-3'
WTIP	5	Forward 5'-CTCTACCGTCTCCCCTGCTC-3'
		Reverse 5'-GACACATGCCTGCCCAAG-3'
PSG6	3	Forward 5'-AGGCAGCCTCACAATCACTG-3'
		Reverse 5'-TGGCCAGCTTTGATGTTTCAGG-3'

Table 2. Primer sequences used for variants validation

RESULTS

1. FAMILY 1

1.1 Clinical findings

- *II-1*

The index case (fig.1) is one of the dizygotic female twins born to non-consanguineous parents. After a normal pregnancy, a caesarean section was performed for placental abruption.

Developmental milestones were appropriately met but at the age of 3 the proband started to have trouble running and fell often. Her weakness in leg muscles then progressed with worsening difficulty at climbing stairs while upper limbs were slowly involved throughout the years.

At the age of 8 myopathic changes, variation in fiber size and internal nuclei were disclosed by a muscle biopsy. Immunofluorescence analysis for dystrophin, α β γ and δ sarcoglycans, caveolin-3 was negative, but highlighted a strong reduction of α -dystroglycan. Immunoblotting showed absence of alpha dystroglycan, and normal levels of calpain and dysferlin. Serum creatine kinase was elevated at 2500 UI/L (normal range <200). A diagnosis of minicore congenital muscular dystrophy was then given on clinical grounds. At 14 yrs the patient loss of ambulation and was not present cardiac involvement.

At last examination the patient used a wheelchair as she lost ambulation at the age of 14. She was able to sit without any assistance for long periods of time, to raise her head from the pillow in the supine position and to make limited movements of her upper limbs upward and laterally. She had joint ankylosis of the knees, ankles, hips, elbows, wrists and reduced pronation of the forearm. Egen Klassifikation score was 11/51. Muscle strength was impaired mainly at shoulder girdles, hip flexors and knee extensors.

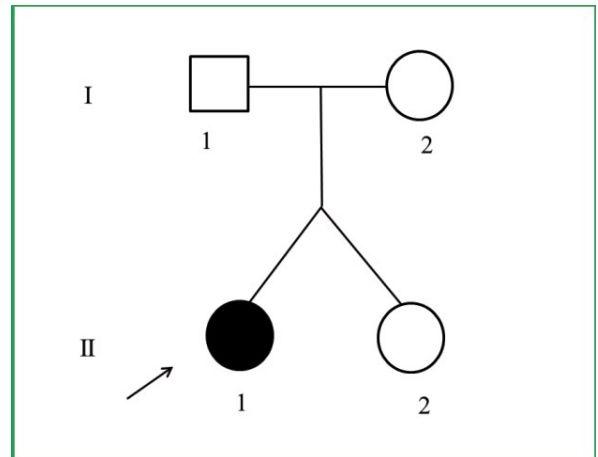


Figure 1. Genealogic tree of family 1

1.2 Exome sequencing analysis

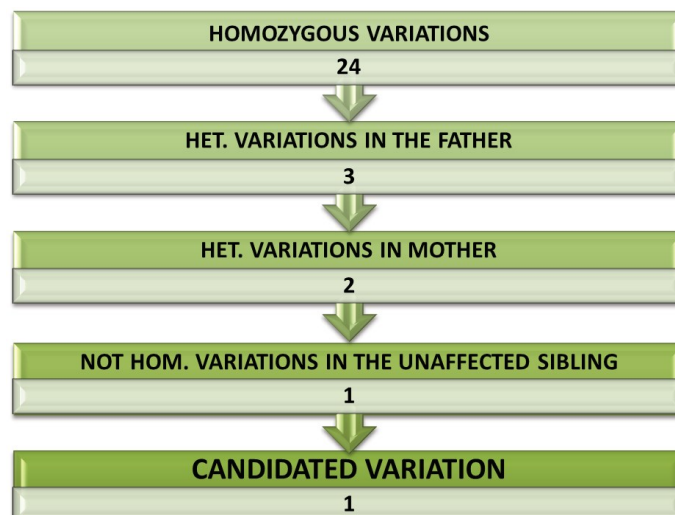
Family 1 was previously screened by Sanger sequencing for *GMPPB* (GDP-Mannose Pyrophosphorylase B), *B3GALNT2* (Beta-1,3-N-Acetylgalactosaminyltransferase 2), *TMEMB* (transmembrane protein B) and *ISPD* (Isoprenoid Synthase Domain Containing) genes, all resulting negatives.

Interrogation of WES resulted in a total of 6855 variations (5416 in heterozygosis-1439 in homozygosis). Vep consequence filter reduced the number of variation to 610 (582 in heterozygosis and 24 in homozygosis).

Prioritization of homozygous variants applying recessive model revealed an in-frame deletion in the known causative *ISPD* gene. The variation segregate with the phenotype: in fact it was found in heterozygosis in the members not affected and in homozygosis in the index case (II-1), (fig.2). The mutation is located on chromosome 7p21.2 (chr.7:16298017; exon 9; c.(1114-1116,964-966)_AAAC>A p.(322,372)_V>-), and was reported as pathogenic variant (Cirak et al. 2013), (fig.3).

ISPD gene contains 10 coding exons and it spans for 334 kb (MIM*614631). The protein encoded by *ISPD* gene belongs to the family of 4-diphosphocytidyl-2C-methyl-D-erythritol synthases (also known as 2-C-methyl-D-erythritol 4-phosphate cytidyltransferases) and is required for efficient O-mannosylation of alpha-dystroglycan (DAG1). Specifically, *ISPD* is thought to aid in the addition of chains of sugar molecules to α -dystroglycan through a process known as glycosylation, critical post-translational modification important for the correct function of α -dystroglycan.

a)



b)

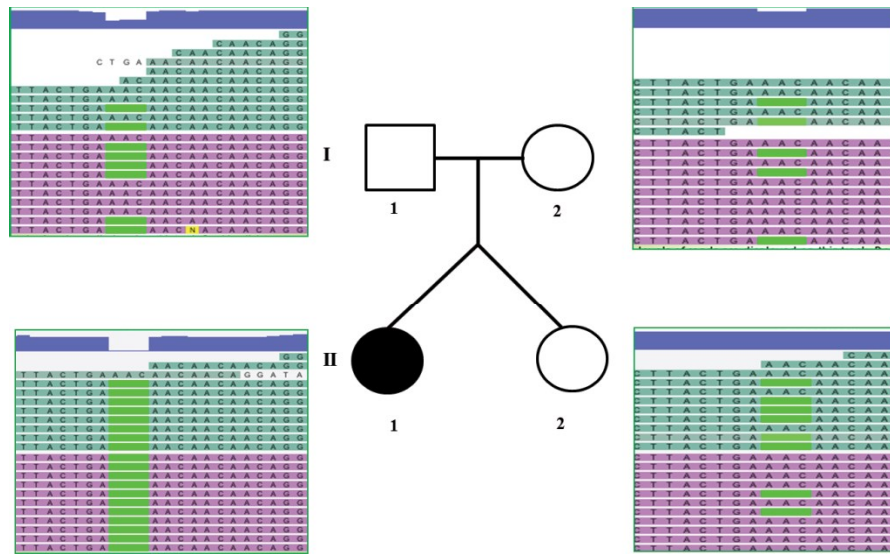


Figure 2. a) Variant prioritization with Clinical Sequence Miner; b) In green the segregation of the identified variation in the pedigree: heterozygous in parents and dizygotic twin, homozygous in the index c

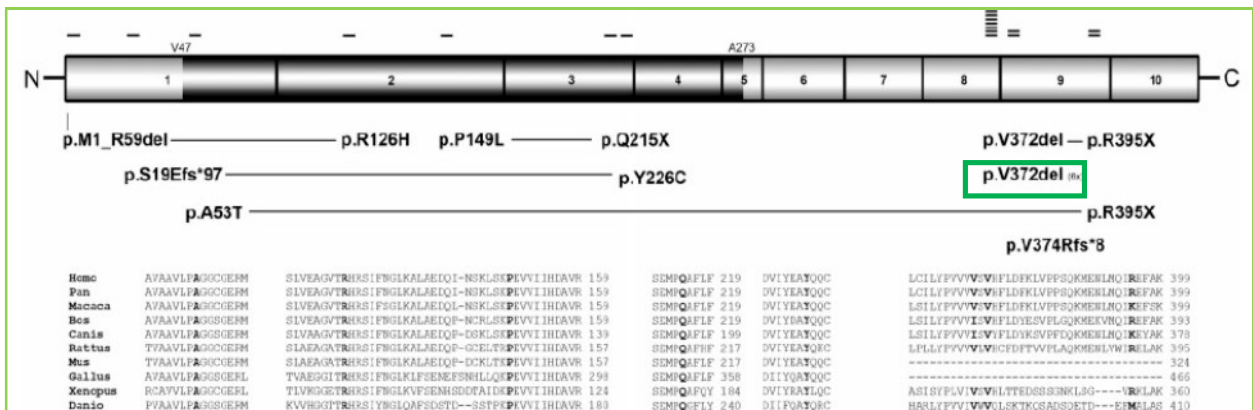


Figure 3. ISPD protein and mutation. The domain structure and exons are illustrated. Dark colour represent the active domain entirely conserved (CDP-ME domain; V47-A273). Rectangle in green indicates localization of our mutation. Horizontal lines indicate mutations found in a compound heterozygosis. The small-sized horizontal bars represent the number of observed alleles. Bottom: conservation of the missense mutations (marked in bold) within the vertebrates: *Homo sapiens* and *Pan troglodytes* 99% amino acid identity; *Macaca mulatta* 94%; *Bos Taurus* 81%; *Canis lupus familiaris* 84%; *Rattus norvegicus* 73%; *Mus musculus* 74%; *Gallus gallus* 66%; *Danio Rerio* 47% (adapted from Cirak et al., 2013).

2. FAMILY 2

2.1 Clinical findings

- *II-1*

The second family (fig.4) consists of two affected siblings born to non-consanguineous parents. There is no family history of neuromuscular, ophthalmological or early-onset heart diseases.

Proband II-1 was a product of a normal pregnancy and normal delivery. As an infant he reportedly had poor suck. He was then evaluated for delayed walking without support (17 months) and toe walking, clumsiness, frequent falls and mild muscle weakness.

At the age 4, a muscle biopsy demonstrated findings typical for congenital muscular dystrophy with type 1 fiber predominance and hypotrophy of some of them». No abnormalities were found on immunoblotting analysis of dystrophin, alpha-sarcoglycan, calpain-3 and dysferlin and on immunohistochemical staining for caveolin-3.

At the time of last follow-up visit, the boy complained of morning myalgia, partially relieved by carnitine, and developed pain after a long walk. He had an elongated face, a high-arched palate, slightly hypotonic facial muscles, hypernasal speech, mild Trendelenburg gait and scapular winging, climbed stairs with unilateral support, couldn't walk on the heels, used a one-handed Gowers maneuver to stand up from the floor, jumped on two legs only. Muscle tone was normal. Muscle strength assessed by the Medical Research Council (MRC) scale was 4 out of 5 in all tested regions but the shoulder and pelvic girdles (3-4/5). Cardiac and respiratory functions were normal and CPK level was 294 UI/L.

- *II-2*

Proband II-2 (fig.4) was born after an uneventful pregnancy. Her parents reported poor suck and frequent deglutition problems. She attained independent walking at 18 months, with unsteadiness and frequent falls but no toe walking. Speech and language therapy was started in order to correct a specific delay. Further motor milestones were acquired slowly, with no regression.

Salient findings at last medical follow-up were pain

in the calves when climbing down the stairs, long face, high-arched palate, slightly hypotonic mimetic muscles, hypernasal speech, mild waddling gait with minimal widening of the base,

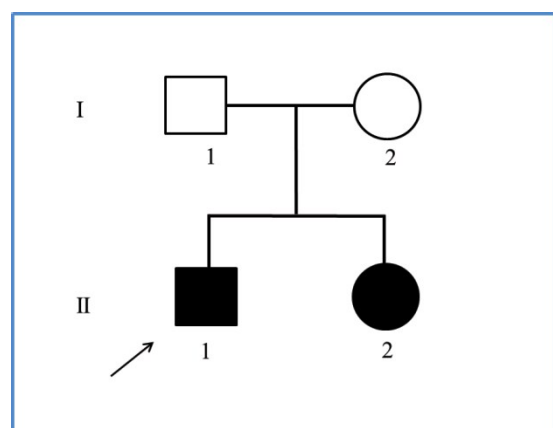


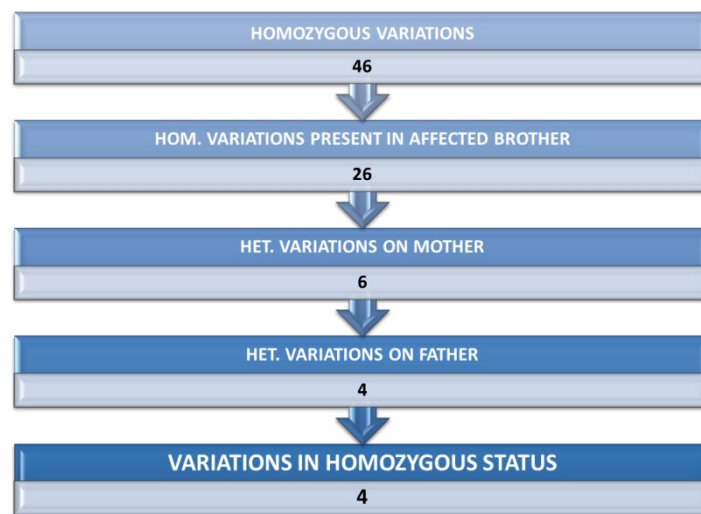
Figure 4. Genealogic tree of family 2

climbed stairs with one-hand support, couldn't walk on the heels, used a partial Gowers maneuver to rise from the floor, jumped on two legs only. She also had mild lumbar hyperlordosis and rigidity. Scapular winging and a reduced range of motion of the ankle joints was noted. Muscle strength was 4 out of 5 (MRC scale). Cardiac function was normal. CPK level is 344 UI/L. No muscle biopsy was available.

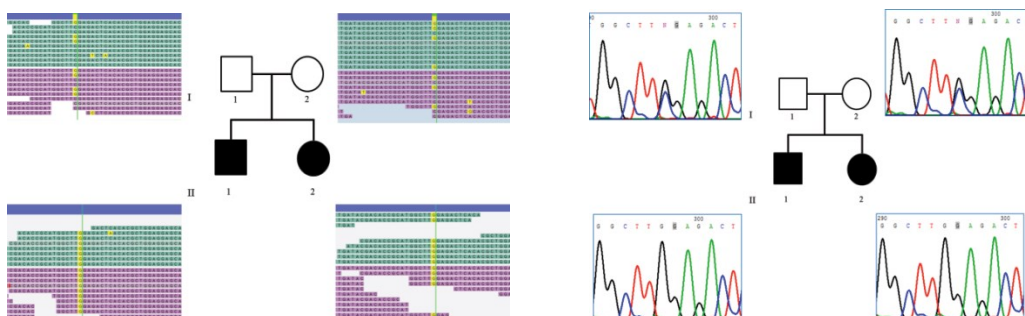
2.2 Exome sequencing analysis

We identified a total of 6913 variations in index case (5440 in heterozygosity and 1473 in homozygosity). Application of vep consequence filter reduced the number of variations to 571(525 in heterozygosity and 46 in homozygosity). Prioritization of homozygous variants applying recessive model (fig.5) resulted in 4 variation shared by the affected siblings and segregating from the parents.

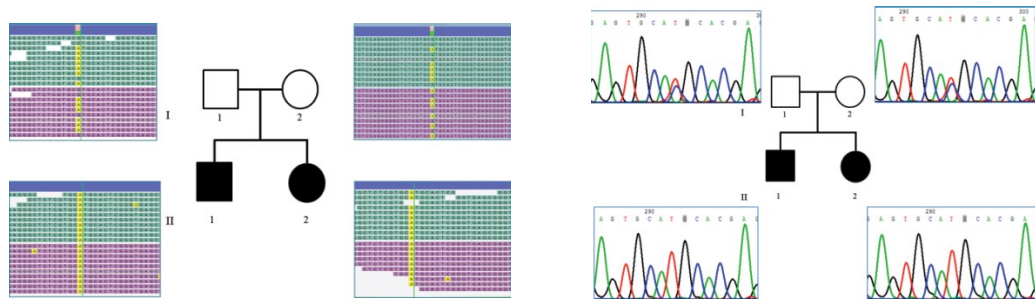
a)



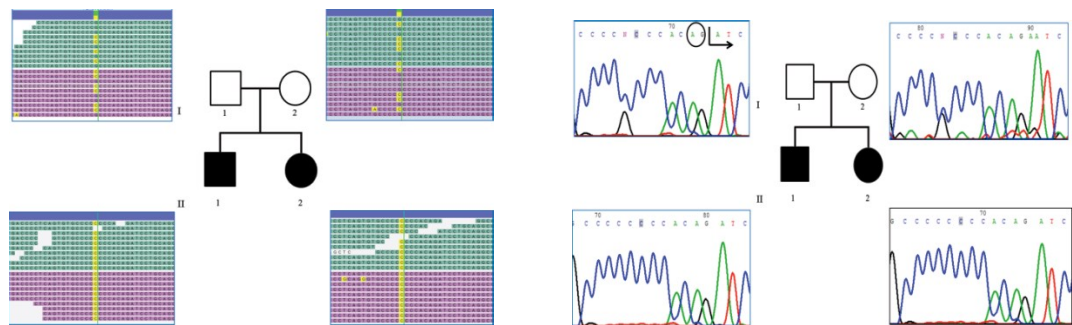
b)



c)



d)



e)

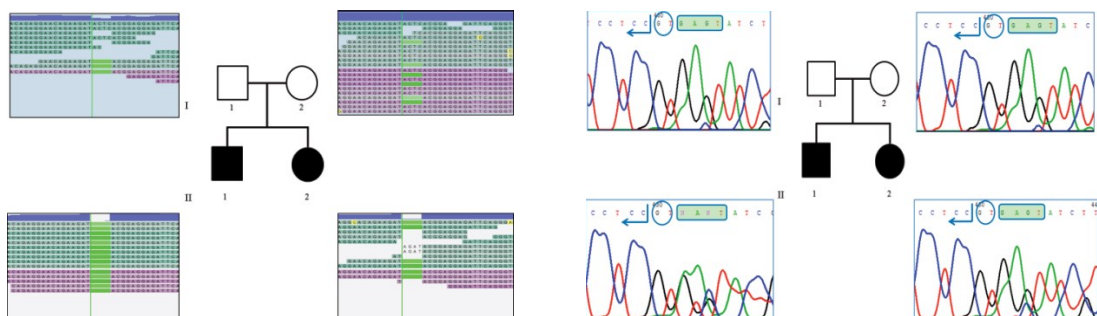


Figure 5. a) Variant prioritization with Clinical Sequence Miner; b) WES output for *RYR1* gene, c) for *ATP4A*, d) for *WTIP*, e) for *PSG6*. In yellow the segregation of the identified variation in the pedigree (heterozygous in parents and homozygous in index case and his sister) and technical validation with Sanger method.

We identified two missense variations in *RYR1* gene (Ryanodine Receptor, Skeletal Muscle; MIM*180901), (chr19:39076790; c.14928 C>G; p.F4976L) and *ATP4A* gene (H⁺,K⁺ Exchanging, Alpha Subunit; MIM *137216), (chr19:36051322; c.730C>T; p.H244Y).

Other two variations are classified as splice region variants in *WTIP* (Wilms Tumor 1 Interacting Protein, MIM*6147905), (chr19:34984389 G>C-8) and *PSG6* (Pregnancy Specific Beta-1-Glycoprotein 6, MIM*176395), (chr19:43414726_43414729delACTC+3). These are summarized in Table 1. All the variants were validated by Sanger sequencing; however the Sanger methods was not able to detect *PSG6* variant (fig. 5).

CHR	GENE	POSITION	REF	CALL	INDEX	CASE	FATHER	MOTHER	FUNCTION	AA	PREDICTION
19	WTIP	34984389	G	C	hom	hom	het	het	splice region	-	polymorphism
19	ATP4A	36051324	G	A	hom	hom	het	het	missense	H244Y	DAMAGING
19	RYR1	39076790	C	G	hom	hom	het	het	missense	F4976L	DAMAGING
19	PSG6	43414725	TACTC	T	hom	hom	het	het	splice region	-	polymorphism

Table 1. In this table are indicated chromosome position, gene, genomic location, reference base, genotype of each family member, functional impact, amino acid substitution and pathogenic prediction of variant identified in each candidate gene.

Pathogenicity of each variant was evaluated through the use of three bioinformatics tools: MUTATION TASTER, SIFT and POLYPHEN-2. MUTATION TASTER set a pathogenicity for *RYR1* and *ATP4A* variants, but not for *WTIP* and *PSG6* that are predicted as polymorphisms. This prediction was confirmed by SIFT, even if the variation in *ATP4A* was reported as damaging with low confidence. No pathogenic prediction was reported by SIFT and POLYPHEN-2 about the variations in *WTIP* and *PSG6* genes. The variant present in *WTIP* is a polymorphism and it is reported with a frequency data in dbSNP (rs45507094, G=0,980/C=0,020) and NHLBI Exome Sequencing Project (<http://evs.gs.washington.edu/EVS/>). According to pathogenic prediction of MUTATION TASTER for *RYR1* and *ATP4A*, POLYPHEN-2 classify the protein changes as probably damaging with a score of 0.98 (*RYR1*) and 0.97 (*ATP4A*).

In the first step we focused our attention on *RYR1* and *ATP4A*, because they showed a missense variation. In a second step, looking at gene function, only *RYR1* was involved in muscle function. *RYR1* gene is located in q arm of the chromosome 19 (19q13.2) and contains 106 exons, and 2 are alternatively spliced.

Clinical presentation of affected patients suggesting an involvement of *RYR1* gene, but the previous screening of this gene resulted negative. Unfortunately the *RYR1* gene was previously screened in the index case for hot spot regions only, and the variation identified by WES falls outside (fig. 6).

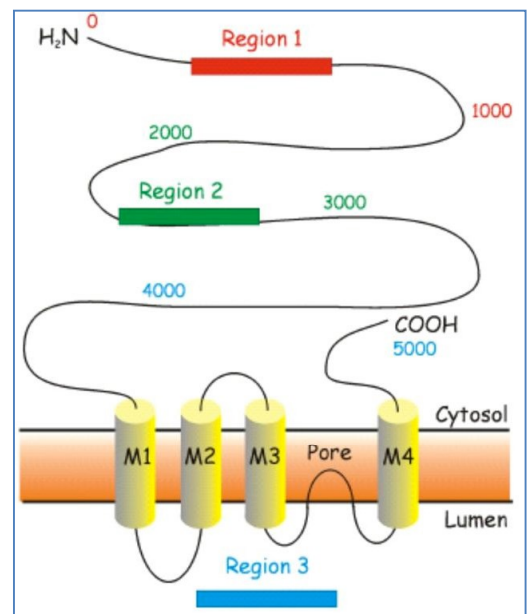


Figure 6. Schematic diagram for the location of the hotspot regions (1-3) and the predicted transmembrane domain (M1-M4) of *RYR1* (Ziober et al., 2010).

The length of the gene was estimated to be approximately 160 kb and the protein length is 5038 amino acids. *RYR1* gene encodes the *Skeletal Muscle Ryanodine Receptor*, which serves as a calcium release channel of the sarcoplasmic reticulum as well as a bridging structure connecting the sarcoplasmic reticulum and transverse tubule.

RYR1 protein is present in the cell as homotetramer with molecular weight of >2MDa (each subunit is >550 kDa), and can also form heterotetramers with RYR2.

RYR1 can also mediate the release of Ca^{2+} from intracellular stores in neurons, and may thereby promote prolonged Ca^{2+} signaling in the brain and seems that is required for normal embryonic development of muscle fibres and skeletal muscle, and normal heart morphogenesis, skin development and ossification during embryogenesis. Its calcium release channel activity is modulated by phosphorylation and resides in the C-terminal region, while the remaining part of the protein constitutes the 'foot' structure spanning the junctional gap between the sarcoplasmic reticulum (SR) and the T-tubule.

The C>G variation causes amino acid change from phenylalanine to leucine at position 4976 and the change involve two hydrofobic amino acids, aliphatic and aromatic respectively.

The variation resides in C-terminal region of the protein and the phenilalanine residue was conserved during evolution between various species (human, mouse, pig, rat, rabbit).

3. FAMILY 3

3.1 Clinical findings

- *II-1*

The disease started in patient “older sib” (fig.7) in his early thirties with steppage gait, inability to walk on tiptoes, lower limb weakness, hypotrophic calves and muscle cramps. A muscle biopsy suggestive of chronic neurogenic atrophy (denervation atrophy plus grouping of monotypic reinnervated myofibres) was performed shortly after disease onset. Brain and spine MRI was unremarkable, with minimal tonsillar ectopia and asymptomatic lumbar and cervical disc protrusions. CPK levels were slightly elevated (303-478 UI/L). Blood tests and targeted analysis of MPZ, PMP22 and AR genes were negative.

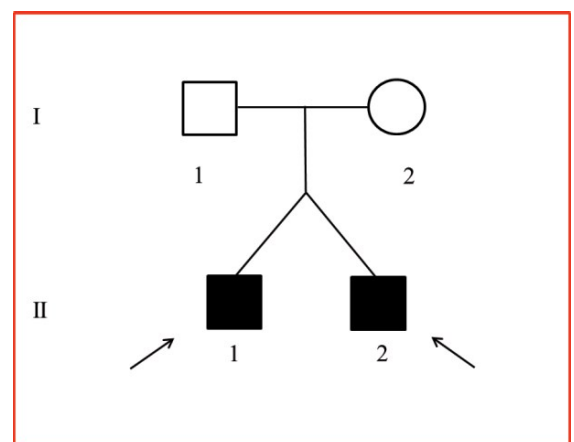


Figure 7. Genealogic tree of family 3

- *II-2*

Since he was 27, patient “younger sib” (fig.7) suffered from worsening disequilibrium, walking and foot dorsiflexion impairment, muscle cramps and weakness, hypoesthesia of lower limbs and paresthesias. He also had irritable bowel syndrome (since the age of 17), bradycardia, retrograde ejaculation (since the age of 34), and lumbar radiculopathy.

Main neurological findings were steppage gait, difficulty walking on tiptoe, loss of deambulation on the heels, very mild muscle atrophy of the hands, reduced muscle strength and tactile sensitivity of the legs and normal deep tendon reflexes, except for absent ankle jerk. Other physical findings included anejaculation likely due to incompetence of the bladder neck and a bladder diverticulum.

An early electromyography revealed lower limb motor axonal neuropathy, confirmed seven years later by a repeat EMG, along with severe denervation of the anterolateral compartment of the leg.

A delayed postural blood pressure response associated with orthostatic intolerance was recorded as measures of autonomic function were taken. Latency of motor evoked potentials were slightly longer than expected for nerve fibers to the left side of the body. Magnetic resonance imaging of the brain, electroencephalography, peripheral nerve ultrasound and electrocardiography were reported as normal.

Histopathological specimens showed a pattern of chronic polyneuropathy. CPK levels were mildly elevated (415 IU/L, normal range <190). Other laboratory investigations were noncontributory: non-significantly increased voltage-gated potassium channel antibodies, normal alpha galactosidase, normal serum B12 and folate, normal routine biochemistry including liver renal and immune function, normal CSF fluid analysis, no antiganglioside IgM antibodies and normal hexosaminidase A activity. Molecular testing for GJB1, PMP22, MPZ, MFN2, HSPB1, HSPB8 and AR genes was negative.

3.2 Gene panel test analysis

We studied all members of family 3 using a lower motor neuron diseases gene panel. The panel includes 21 ALS- 13 SMA -16HMN- and 14 CMT-associated genes (see table 1 in Materials and Methods).

This analysis allowed us to identify a hemizygous missense mutation in exon 15 of the X-linked *ATP7A* gene (c.2972 C>A, p.A991D), in both affected brothers. The same variant was present in heterozygosis in the mother not affected.

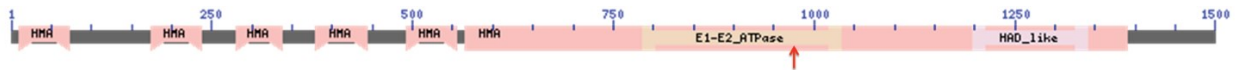


Figure 8. ATP7A gene map. The red arrow indicates the position of variation identified.

In silico evaluation of the variant (MUTATION TASTER, POLYPHEN2), its absence from EVS, and the compatibility of the pedigree with an X-linked recessive segregation all support the pathogenic nature of this mutation.

ATP7A gene (ATPase, Cu⁺⁺transporting, alpha polypeptide), localized on Xq21.1 (fig.8), encodes a transmembrane copper transporter P-type ATPase and controls cellular copper homeostasis.

The copper is a cofactor of several enzymes, useful energetic cellular metabolism, iron metabolism and collagen maturation.

May supply copper to copper-requiring proteins within the secretory pathway, when localized in the trans-Golgi network. Under conditions of elevated extracellular copper, it re-localized to the plasma membrane where it functions in the efflux of copper from cells.

Its catalytic activity is the following: $ATP + H_2O + Cu^+(Side\ 1) = ADP + phosphate + Cu^+(Side\ 2)$.

The gene is constituted by 23 exons and it spread for 150 kb of genomic DNA. The protein consists of 1500 amino acids and its molecular weight is 163,334 kDa, and contains eight transmembrane domains (N-terminal and C-terminal are located in the cytosol), (fig.9).

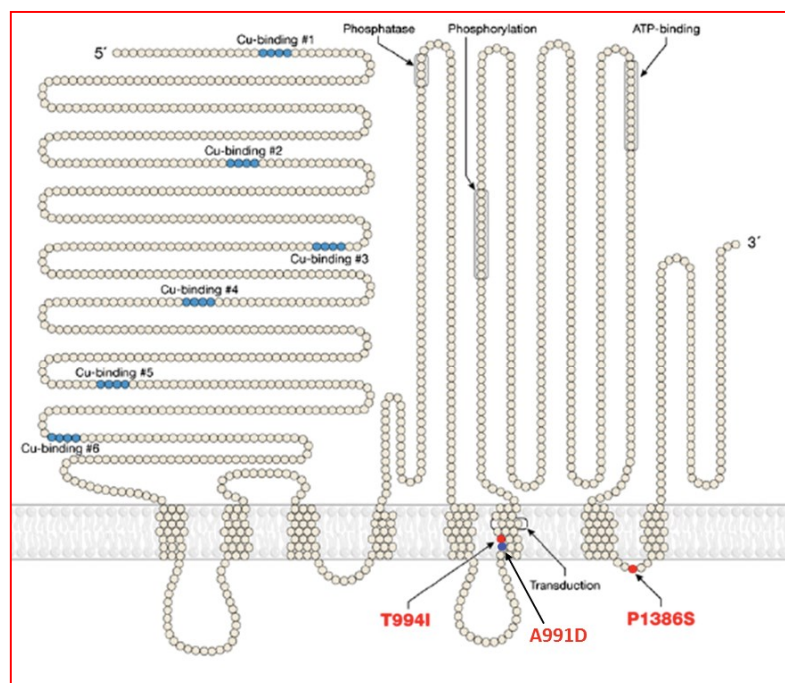


Figure 9. ATP7A protein domains. Blue circle indicates localization of mutation found in affected brothers of family 3. Red circles represent the mutations found in *ATP7A* gene and associated to X-linked distal motor neuropathy (adapted from Kennerson et al., 2010).

The effect of variation is represented by amino acid change alanine/aspartic acid within the sixth transmembrane domain. At biochemical level this means that happens a substitution between an hydrophobic and aliphatic (Ala) with a hydrophilic amino acid.

DISCUSSION

In my work I studied, three “families of four” presented, a clinical phenotype suggestive of congenital muscular dystrophy, congenital myopathy and hereditary distal motor neuropathy with next generation sequencing technologies (WES/Gene Panel) to identify disease causing genes.

- Congenital muscular dystrophies are a group of clinically and genetically heterogeneous neuromuscular disorders with onset at birth or in infancy, congenital contractures, and immunohistochemical findings of dystrophic changes on muscle biopsy. The patients affected by CMD show hypotonia, weakness and the typical presentation of “floppy baby”.

One of the major pathological subgroups is characterized by a reduction in the functional glycosylation of aDG (aDGpathies). Mutations in eight genes encoding for glycosyltransferases or other proteins involved in α -dystroglycan glycosylation pathway are responsible for 50–60% of the cases classified as dystroglycanopathies (Godfrey et al., 2007; Mercuri et al., 2009; Messina et al., 2010; Devisme et al., 2012; Cirak et al., 2013). However many causative genes remain undiscovered.

In order to identify a causative disease gene, we analysed four individuals (one affected and three healthy) of the CMD family with autosomal recessive inheritance. The result obtained by analysis of exome by WES indicated unique candidate gene in *ISPD* (Isoprenoid Synthase Domain Containing; [MIM*614631]).

ISPD is located on chromosome 7 and the protein belongs to the family of 4-diphosphocytidyl-2C-methyl-D-erythritol synthases (also known as 2-C-methyl-D-erythritol 4-phosphate cytidyltransferases) and is required for efficient O-mannosylation of alpha-dystroglycan (DAG1), Dystroglycan is composed of two subunits, α and β ; a post-translation cleavage produced a transmembrane protein (α) and an external protein (β) which is heavily O-glycosylated, modification that is critical for correct interaction with extracellular matrix proteins (laminin, agrin, perlecan, neurexin and pikachurin), (Kanagawa and Toda, 2006; Waite et al., 2012; Cirak et al., 2013). Without a functional α -dystroglycan to stabilize muscle cells (as in the WWS), muscle fibres are damaged during the repetitive cycles of contraction and relaxation with use, weaken and die over time, affecting the development, structure, and function of skeletal muscles.

The protein is ubiquitously expressed with high expression in brain (Willer et al., 2012). Defective α -dystroglycan affects the migration of neurons during the early development of the

brain, causing severe brain abnormality called cobblestone lissencephaly, in which the surface of the brain lacks the normal folds and grooves and instead appears bumpy and irregular.

It has been observed that generally diseases caused by mutations in *ISDP* are progressive, with many cases characterized by LGMD losing ambulation during their early teenage years, thus following a Duchenne muscular dystrophy-like path. Also several patients manifest pseudohypertrophy, including the tongue and a deterioration of respiratory and cardiac functions (Mercuri et al., 2003; Murakami et al., 2006; Bourteel et al., 2009; Lefeber et al., 2011; Yilmaz et al., 2011; Cirak et al., 2013).

In 2009 Cirak and colleagues (Cirak et al., 2009) using traditional positional cloning discovered a new locus in a family affected by congenital muscular dystrophy without brain involvement mapping to chromosome 7p21. More recently were discovered bi-allelic loss of function mutations in *ISPD* causing Walker Warburg Syndrome (Willer et al., 2012; Roscioli et al., 2012). Following these works Cirak et al. analysed the full phenotypic spectrum caused by recessive mutations in *ISPD* gene, and they observed that it ranges from congenital muscular dystrophy without central nervous system (CNS) involvement, milder cases with LGMD without CNS involvement to a novel phenotype with relatively mild skeletal muscle weakness and structural brain involvement. They noted that missense mutations manifesting in compound heterozygosity and a loss of function mutations affecting the first 5 exons results in a congenital muscular dystrophy, LGMD-CRB (cerebellar involvement) or LGMD with mental retardation. In fact the first five exons encode for functional conserved 4-diphosphocytidyl-2-methyl-D-erythritol synthase (CDP-ME) domain. The cases found by Cirak et al. with milder LGMD without brain involvement show either a homozygous in-frame deletion of a single amino acid or are compound heterozygous mutations.

In our family was reported an homozygous in-frame deletion of a single amino acid (p.V372del) in exon 9, the same mutation found by Cirak et al. The female affected showed elevated serum creatine kinase level, loss of ambulation at 14ys, weakness at lower limbs, frequent falls, progressive weakness at the upper limbs, difficulty at climbing stairs, muscle strength impairment mainly at shoulder girdles, hip flexors and knee extensors, and finally a strong reduction of α -dystroglycan at immunostaining analysis. We conclude that there is a good genotype/phenotype correlation in our family presented with mutation in *ISPD* gene.

The pathogenic effect of mutations in *ISPD* could be explained by the putative function of *ISDP* in eubacteria, green algae and chloroplast of higher plants. In these organisms *ISPD* orthologues are involved in the methylerythritol pathway that is absent in mammals and humans. For this reason *ISPD* must be involved in different functions in humans (Cirak et al. 2013).

The mechanism underlying the reduction of α -dystroglycan caused by ISPD has not yet been discovered. It has been observed that in patients with ISPD-deficient Walker–Warburg syndrome, O-mannosylation was strongly impaired and subsequently the O-mannosyl phosphorylation and LARGE-dependent hyperglycosylation were absent (Willer et al., 2012; Cirak et al., 2013). This indicates that ISPD should be implicated in the initial step of glycosylation of α -dystroglycan.

Recently in another work was reported a homozygous mutation in a family of Pakistani origin with 2 cousins from consanguineous parents affected by CMD/early LGMD intermediate phenotype and a CMD, respectively. The variation occurs in exon 2 (Gly123Arg) and falls into a highly conserved region of the protein that contains the functional conserved 4-diphosphocytidyl-2-methyl-D-erythritol synthase (CDP-ME) domain. Differently from the cases reported by Roscioli et al. and Cirak et al., in the last work the homozygous missense mutation is associated both with a milder CMD/early LGMD intermediate phenotype without brain involvement or mental retardation, and with a more severe CMD phenotype (Baranello et al., 2015). In summary genotype–phenotype correlation may not be straightforward in patients with mutations in ISPD.

- Congenital myopathies (CM) define a group of heterogeneous disorders characterized by early onset (neonatal or childhood) progressive or no progressive muscle weakness, breathing difficulties and delayed motor milestones. Classification is based on recurrence of typical histological anomalies on muscle biopsy. Many causative genes have been identified in the last decade (more than 20 genes), underlying the high genetic heterogeneity of these pathologies; however only a small proportion of patients to date receive a genetic molecular diagnosis.

We have studied a CM “family of four” with autosomal recessive inheritance composed by two affected kids (one male and one female) and healthy parents. The index case (male II-1) was affected by congenital myopathy, delay of psychomotor development milestones, retraction of the ankles and finger's flexors and hypotonia of mimic muscles. The sister had the same clinical presentations. WES data were processed to obtain homozygous variations and we identified a missense mutation in the *RYR1* gene (Ryanodine Receptor, Skeletal Muscle), which was compatible with the patient's phenotype and it was never reported.

Ryanodine receptors (RyRs) represent the sentinels of massive intracellular calcium stores contained within the sarcoplasmic reticulum (Capes et al., 2011), and in response to the sarcolemma depolarization of T-tubules, RyRs mediates the release of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm and thereby plays a key role in facilitating

mobilization of the myofilaments and triggering muscle contraction. In mammals are expressed three isoforms encoded by three distinct genes (*RYR1*, *RYR2*, *RYR3*) residing on separate chromosomes. The first isoform is expressed predominantly in fast and slow twitch skeletal muscle, and at lower levels in gastric smooth muscle, B lymphocyte and in cerebellar Purkinje cells. RyR2 is the major isoform present in cardiac muscle and it is robustly expressed in neurons, in visceral and arterial smooth muscle. RyR3 seems to play a key role during development, although in mature cells is found in brain, smooth muscle, epithelial cells and diaphragm. All three isoforms show 65% of amino acid identity (Capes et al. 2011).

Many cytosolic metabolites and proteins within the lumen of the sarcoplasmic reticulum and those associated directly with the receptors participates to accurate regulation mechanism of Ca^{2+} release-sequestration cycle, as for example: the dihydropyridine receptor (DHPR), various ions such as Mg^{2+} , Ca^{2+} , protein kinase A (PKA), FK506 binding protein (FKBP12), calmodulin (CaM), Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), calsequestrin (CSQ), triadin, junction. Mg^{2+} and ATP modulate activity of RyRs inner the cytoplasm and Ca^{2+} regulates RyRs both in the cytoplasm and in the lumen of SR. In addition RyRs have important function in signal transduction in osteoclasts and in nervous system, where contributes to synaptic plasticity, learning, apoptosis and secretion (Zaidi et al. 1992; Chavis et al. 1996; Schwab et al. 2001; Lanner et al., 2010).

Both dominant and recessive mutations have been reported in *RYR1*. Dominant mutations have been classically associated with central core disease (CCD; [MIM*117000]), (Lynch et al., 1999; Monnier et al., 2001; Davis et al., 2003) in 90% of cases and/or susceptibility to malignant hyperthermia (MH; [MIM*145600]), (Robinson et al., 2006), while recessive mutations predominate in multimincore disease (MmD; [MIM*255320]), (Jungbluth et al., 2007), centronuclear myopathy (CNM; [MIM*160150]), (Wilmshurst et al., 2010), and congenital fibre type disproportion (CFTD; [MIM*255310]), (Clark et al., 2010).

In the Central Core disease type I muscle fibres are preferentially affected with the central areas characterized by disrupted myofibrillar architecture with sarcomeric disorganization, absence of mitochondria and their oxidative enzymes (Rendu et al., 2013; Capes et al., 2011). A potential cause of mitochondrial impairment (swelling and death) should be probably due to an excess of Ca^{2+} in the central area of the fibre much more vulnerable to Ca^{2+} gradients (Locke et al., 1998; Capes et al., 2011). A second theory of core formation would be based on a study using knock-in mouse expressing in heterozygosis a leaky-channel mutation in the RYR1 N-terminal region (Durham et al., 2008; Capes et al., 2011). They proposed that oxidative stress, proteolysis and stretching of myofilaments due to prolonged contracture in presence of unsequestered Ca^{2+} ,

should cause a deterioration of the fibres and core formation and malignant progression of disease.

MH is a pharmacological disease triggered by inhalational anesthetics (e.g. halothane) or depolarizing muscle relaxants (e.g. succinylcholine). MH episodes are rare, about 1 in 10.000 surgeries and susceptible individuals develop muscle rigidity, arrhythmias, hyperkalemia, respiratory and metabolic acidosis, and an increase in body temperature and a fulminant death occur unless a decrease of body temperature.

Multiminicore disease (MMD) main features include neonatal hypotonia, delayed motor development, and generalized muscle weakness and amyotrophy, which may progress slowly or remain stable. Muscle biopsy shows multiple, poorly circumscribed, small areas of sarcomere disorganization and mitochondria depletion (areas termed minicores) in most muscle fibres.

Moreover defects in *RYR1* are a cause of congenital myopathy with fibre-type disproportion (CFTD), characterized by a relative hypotrophy of type 1 muscle fibres compared to type 2 fibres on skeletal muscle biopsy.

Dominant mutations clustered in three hot spots: N-terminus (Cys35- Arg614) and central region (Asp2129-Arg2458) mainly associated with MH, and C-terminus (Ile3916-Ala4942), (Capes et al., 2011; Amburgey et al., 2013) associated with CCD. It has been hypothesized that dominant mutations associated with MH could RYR1 hyper-excitability, while associated mutations with CCD could result in chronic channel dysfunction, either through excitation-contraction uncoupling or by persistent channel leakiness (Treves et al., 2008; Amburgey et al., 2013).

However much less is known about the mechanisms of recessive mutations to the contribution of disease phenotype. The largest study was performed by Klein and colleagues in 2012 on 36 families with recessive inheritance. In summary they observed that patients with recessive mutations manifested with much early onset severe clinical presentations, more widespread important weakness and more involvement of extra ocular and bulbar musculature (Klein et al., 2012).

Recently a smaller study has been identified by WES recessive mutations in *RYR1* in six patients with clinically different skeletal muscle pathologies, ranging from a fatal neonatal myopathy to a mild and slowly progressive myopathy with adult onset. They identified five missense mutations spanning in various exons confirming there is no precise hot spot region or genotype-phenotype correlation for recessive mutations.

Even in our patients can be observed a mild/slowly progressive myopathy, but with no severe early onset and at the muscle biopsy analysis our patients, as in the work of Böhm et al. they present type 1 fibre predominance.

To date the largest examination of recessive cases was carried out by the work of Amburgey and colleagues. They studied 106 cases with recessive mutations in *RYR1*, and the most highly represented were the core myopathies (51%) (inclusive of MmD, CCD, atypical-core myopathy, core/rod disease), and CNM/CNM like myopathies (23.6%). The first important finding that the work highlight is the association between increased disease severity and the presence at least one hypomorphic allele (i.e. non sense, frame shift, splice site). This data suggests that reduced levels of RYR1 protein are involved in more severe disease manifestation. In particular the majority of patients with CCD (based on histopathologic diagnosis) had two non-hypomorphic mutations (missense or in frame indel). Patients with only missense or small in-frame indel mutations (and no hypomorphic mutations) showed significantly milder clinical phenotype. Finally they found that missense mutations were generally enriched in the MH/CCD hot spots and specifically enriched in the selectivity filter of the channel pore.

The missense mutation that we identified in the affected siblings is located in the C-terminal and causes an amino acid change from phenylalanine (F) to Leucine (L) and both are hydrophobic amino acids. Most mutations associated with CCD have been identified in this region. Although the change is localized out of C-terminus hot spot region of RYR1 (Ile3916-Ala4942), this protein part comprises the transmembrane/luminal and pore-forming regions of the sarcoplasmic reticulum calcium-release channel and mutations that localize there may directly alter the functions of the channel.

It has been noted that identical *RYR1* mutations cause MH in some human patients and CCD in others. The mechanism(s) that leads to the different phenotypes remains undiscovered. Interesting is that the patients with MH who lack clinical myopathies are more likely to have mutations at the N-terminus of *RYR1*, whereas those with clinical myopathies are likely to have mutations at the C-terminal. On the basis of data literature of clinical presentations, gene function and mutational analysis there is a good correlation between RYR1 pathogenic variant and the clinical phenotype of our two affected patients.

- The distal hereditary motor neuropathies (dHMNs) comprise a clinically and heterogeneous group of disorders primary affecting motor neurons in the peripheral nervous system. Patients affected show progressive muscle weakness and wasting starting in the distal muscles of the limbs without sensory involvement. The disease causes gradual degeneration of motor neuron axons in the limbs, beginning in the distal portion and progressing toward the cell body. To date have been mapped fifteen loci and have been identified eight genes which includes transfer RNA synthetase, two heat shock protein, and a microtubule motor protein involved in

axonal transport. However more than 80% of patients with dHMN have mutations in unknown genes (Dierick et al., 2008; Rossor et al., 2012).

We studied a family of four with two brothers presenting a distal axonal motor neuropathy and a focal autonomic dysfunction, which main manifestation was retrograde ejaculation. One of the two sibs also presented enhanced gastro-intestinal motility and bradycardia. Their clinical findings were highly similar. We performed an NGS analysis for lower motor neuron diseases (LMND Gene Panel) and we found a novel hemizygous missense mutation (p.A991D) in the X-linked *ATP7A* gene; while the unaffected mother showed the variation in heterozygous status.

ATP7A gene (ATPase, Cu²⁺ transporting, alpha polypeptide), localized on Xq21.1, encodes a transmembrane copper transporter P-type ATPase. Copper is required as a cofactor for numerous critical enzymes as cytochrome c oxidase, ceruloplasmin, lysyl oxidase (Lutsenko 2010; Nevitt et al. 2012; Telianidis et al., 2013). *ATP7A* and *ATP7B* together controls cellular copper homeostasis: the first is ubiquitously expressed in extrahepatic cells and tissues, but mainly in the small intestine. This pattern of expression explains the systemic defects caused by its absence or inactivation. The second, has highest expression level in the liver, and lower levels in the kidney, placenta, brain, heart, and lungs (Bull et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993). Classically, mutations in *ATP7A* lead to Menkes disease (MK; [MIM* 309400]), a severe infantile-onset developmental disorder (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993; Kaler et al., 1994) and to its milder variant, occipital horn syndrome with skeletal and skin abnormalities and autonomic dysfunction (OHS; [MIM* 304150]), (Kaler et al., 1994; Tang et al., 2006). The most severely symptoms in Menkes disease are caused by an impaired intestinal copper absorption that lead to systemic copper deficiency and consequently, reduced activity of critical copper-dependent enzymes and includes: abnormal neurodevelopment, seizures associated with cerebral atrophy and dysmyelination, a range of connective tissue and vascular abnormalities, fragile bones, an atypical kinky hair structure (pili torti), hair and skin pigmentation defects and failure to thrive (Danks, 1995; Kaler, 2011). Common features of Occipital horn syndrome are unusual facial appearance, multiple skeletal abnormalities, loose skin, primarily connective tissue defects and moderate neurological symptoms, chronic diarrhea, genitourinary defects.

The reduced levels of *ATP7A* transcript are caused mainly by splice site mutations (Kaler, 2011). The milder phenotype suggests that sufficient residual functional *ATP7A* is produced, but the prominent connective tissue defects indicate that copper delivery to lysyl oxidase is severely disrupted (Kaler, 2011; Scheiber et al., 2013).

A form of dHMN X-linked (Xq13.1-q21) (DSMAX; SMAX3; [MIM*300489]) was reported in a Brazilian family (Takata et al., 2004) and in three generation families (Kennerson et al., 2009). In 2010 was identified *ATP7A* as gene responsible for the spectrum of distal motor neuropathy (Kennerson et al., 2010). They identified two missense mutations, the first in exon 22 predicting p.P1386S amino acid substitution and the second in exon 15 predicting a p.T994I amino acid substitution; both positions are highly conserved across evolution. The variant reported in Brazilian family (p.T994I) is within the same α -helix-TH domain of the novel mutation identified in our family, only 3 residues apart (p.A991D).

Missense mutation occurring in exons 15 appear to be extremely rare and also seem not disrupt critical functional domains or disturb proper splicing or cause reduced levels of *ATP7A* protein. On the contrary Menkes disease is due mainly to deletions, splice-site mutations (at canonical positions), nonsense mutations, missense mutations that affect a critical functional domain in *ATP7A* or induce misfolding, and occipital horn syndrome is associated with leaky splice-junction mutations involving non canonical bases (Kennerson et al., 2010).

It has been observed that dHMNs patients manifest a type of adult-onset of disease because motor neurons might be particularly sensitive to perturbations in copper homeostasis. The phenomenon of late, often adult-onset, distal muscular atrophy implies that these mutations produce subtle effects that require years to triggered pathological consequences. As postulated by Kennerson et al. *ATP7A* mutations probably would reduce, rather than exaggerate, activity of superoxide dismutase; in fact has been noted that familial form of amyotrophic lateral sclerosis are implicated gain of functions missense mutations in this enzyme (Bruijin et al., 2004). It is possible that even a small reduction in activities of certain cuproenzymes might contribute to distal motor neuropathy phenotype.

In our case could be supposed that the amino acid change from hydrophobic to hydrophilic residue could alter the correct localization into the TGN plasma membrane and impair the correct cellular Cu^{2+} homeostasis. At clinical level our patients show main clinical signs of distal motor neuropathy (cramps, progressive difficulties in feet dorsiflexion, lower limbs distal muscle weakness, distal muscle atrophy without sensory involvement); focal autonomic dysfunction; moderate skin laxity; occipital horns at Rx in the younger brother (not determined in the older brother). Both brothers do not manifest the severe infantile central neurological deficits observed in Menkes disease. In addition the SMAX3 phenotype presented in the works of Kennerson et al. encompasses all the symptoms and signs present in two affected brothers; however in SMAX3 patients previously studied a pure axonal distal motor neuropathy was present, but without symptoms of dysautonomia. There is a good genotype-phenotype correlation in that the amount

of residual copper ATPase activity determines the phenotype. In the patient with SMAX3, copper and ceruloplasmin serum concentrations are invariably in the low-normal range.

In conclusion the phenotype we describe in our dHMN family is novel since it includes autonomic dysfunction, skin laxity and occipital horn sign, expanding the ATP7A clinical spectrum, rather than distinct phenotypes.

Final remarks

In our work we studied both CMD and CM families using whole exome sequencing approach. In the first family *ISPD* involvement was missed by previous traditional Sanger sequencing analysis and in the second family the large *RYRI* gene was previously screened in the index case only for hot spot regions. In both cases WES was demonstrated a powerful tool for variation discovery with high accuracy and for refining diagnostic evaluation. In particular, molecular dissection of the entire *RYRI* gene through Sanger methods resulted expensive and time consuming step-by-step process.

In the third family we applied a more specific and targeted gene LMND panel approach that proved as powerful tool for HMN diagnosis and new phenotype discovery.

It is possible to find several advantages in diagnostic exome, as identification of mutations in known genes “correcting” a non-exhaustive diagnostic approach, an accurate molecular diagnostic approach for pathologies with high genetic heterogeneity and/or large genes involved, and differently from gene panels, exploring selected regions, diagnostic exome can discover novel disease genes. On the other hand exome approach presents some disadvantages or technical limitations. In fact not all the exons in the genome are targeted (>97%). Many genomic regions cannot be captured and sequences, including many exons and about 10% of exons that are targeted may not be well covered. Moreover certain mutation types are not detectable, such as large rearrangements, CNVs, mitochondrial genome mutations, trinucleotide repeat expansions, epigenetic effects and after that novel variants/novel disease gene are identified, a complex post-test workflow is needed to prove disease causing significance or variant clinical interpretation.

The advantage in the use of the panels over exome-based approach is that they sequence fewer targets, so in a single run are analysed simultaneously many genes (64 in our case). The technology ensure a certain security and reliability in finding any type of variations and also give much more information in the presence affected and healthy patients when the parents are present. To date panel-based approach can achieve very high levels of coverage ($\geq 99\%$), as well very high sensitivity and specificity for single nucleotide variant detection. Also variant

interpretation of relatively small numbers of potentially novel or pathogenic is much handly (Newman and Black, 2014).

Diagnosis of NMDs disease is very challenging in reason of high genetic heterogeneity (large number of causative genes), phenotypic heterogeneity (association of multiple genes with similar phenotype or single gene with multiple phenotypes), allelic heterogeneity (various number and type of pathogenic variants sparse in the length of each gene), and finally the significantly larger sizes of most NMD genes: *TTN*, *RYR*, *DMD*, *DYSF*, *LMNA* and others (Ankala et al., 2015 b). In addition, the novel oncoming clinical trials and personalized therapies make the genetic diagnosis mandatory in order to be enrolled in such treatments. This makes NGS diagnosis very welcoming for NMDs.

In conclusion WES analysis demonstrates very useful in the study of three “family of four” to discover homozygous pathogenic variants in known genes (*ISPD* mutations in affected patients of CMD family and *RYR1* in two affected siblings in the family with CM) and, on the other hand, application of Gene Panel test to family with hereditary motor neuropathy was able to identify a disease causing gene (*ATP7A*) in patients with heterogeneous phenotype and without a specific diagnosis.

CONCLUSION

The whole exome sequencing analysis on the first family with congenital muscular dystrophy allowed to identify an in-frame deletion in *ISPD* gene reported as pathogenic from literature's data. An accurate prioritization of the variants was conducted to find a unique candidate gene.

In the second family with congenital myopathy variant prioritization allowed the identification of two missense variations (in *RYR1* and *WTIP*) and two splice region variants (in *ATP4A* and *PGS6*).

Pathogenic prediction analysis with bioinformatics tools and analysis of gene function and data literature, referred to *RYR1* as candidate gene.

Interpretation of the biological and clinical significance of *ISPD* and *RYR1* permitted to postulate an appropriate genotype-phenotype correlation and, especially, it was possible offer a correct diagnosis to affected patients. In these two cases, WES identified mutations in two known genes "correcting" a non-exhaustive diagnostic approach. This is an example confirming the usefulness of next generation sequencing as an accurate molecular diagnostic approach for pathologies with high genetic heterogeneity and/or large genes. In both cases, WES has demonstrated to be a powerful tool for variation discovery with high accuracy and, in particular, it demonstrates the importance of clinical evaluation to understand the clinical impact of the variant.

On the third family with distal motor neuropathy it has been conducted a multi-gene panel test approach after evaluation of clinical signs and molecular genetic history of the affected patients. Their clinical presentations included typical signs of X-linked distal axonal motor neuropathy and a focal autonomic dysfunction.

Gene Panel analysis has identified a never reported missense mutation in *ATP7A* gene that resulted as pathogenic by prediction analysis with bioinformatics tools. This allowed a more correctly and precise reevaluation of clinical phenotype, in the light that mutations in *ATP7A* were involved in Menkes disease, occipital horn syndrome and recently associated with a X-linked form of distal motor neuropathy.

The phenotype we describe in our family is novel since it includes autonomic dysfunction, expanding the *ATP7A* clinical spectrum. The utilized NGS clinical genes panel for lower motor neuron diseases is therefore a powerful tool for hereditary motor neuron diagnosis and new phenotype discovery.

Therefore exome approach revealed to be an excellent, and successful tool with high diagnostic power and high rate of accuracy and efficacy and useful in "family of four" studies.

WES and more in general NGS-based approaches are expected to be widely adopted both in research and diagnostics.

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