Final Report Summary - BIO-NMD (Identifying and validating pre-clinical biomarkers for diagnostics and therapeutics of Neuromuscular Disorders)

Executive Summary:

The aim of the BIO-NMD project was the identification of pre-clinical, novel, genomic and proteomic biomarkers, which may correlate with drug response, diagnosis and disease prognosis of the selected NMDs (DMD, BMD, COL6 myopathies) by determining genomic variations as well as defining specific transcription or protein profiles or disease-related pathways both in human and in suitable animal models. In order to realize this ambitious objective the BIO-NMD adopted two main strategies.

The first was the omic approach, meaning analyzing the whole genome, transcriptome, or proteome sets using innovative technologies, as whole exome sequencing, sequencing of the full transcriptome (RNAseq) and proteome profiling, by custom designed multiplexes immunoassays or using novel high throughput techniques on whole proteome.

The second was the more traditional targeted search for biomarkers using candidate genes/proteins selected on the bases on bioinformatics tools enabling gene prioritization based on their functions or relationships with the functional pathways (interactome) which are involved in the selected diseases. In addition, the project, via the clinical task force, identified some patients' categories, to be selectively studied and compared each other.

This novel approach was adopted to overcome the known difficulties in sorting out genome inflation (for SNPs search or massive parallel sequencing) and the need to study highly dimensioned population in order to have results with statistical meaning. This global approach has made possible the identification of biomarkers in a relatively small population as DMD and COL6 myopathies, and based on the analysis of a relatively small number of samples, as usually happens when studying rare diseases. The BIO-NMD was a discovery project but it envisaged a clinical impact through the biological validation of the identified biomarkers in large clinical series.

The project original prevision was to identify 4-6 biomarkers usable for DMD, COL6 disease and perhaps translatable in other rare neuromuscular diseases. In light of what we have experienced and carried on during these three years, our vision is slightly different, but even more forceful. Indeed we have discovered many exploratory biomarkers; all of these were technically validated using different independent methods/platforms. Among these we have prioritized biomarkers based on multiple evidences and specific interactome characteristics.

The final number of biomarkers we have discovered and that should be further exploited and biologically validated in other patients cohort is higher since we have learned that for rare diseases (and probably not only) a biomarker reflects a very specific aspect of the disease, or a fine tuned clinical feature, as age at loss of ambulation, corticosteroid response, maintaining (loosing of certain clinical ability (respiratory capacity) or occurrence of specific clinical signs, as dilated cardiomyopathy. These biomarkers allow us to distinguish between specific phenotypes.
In these terms, we do have now many biomarkers, either proteomics or genomics, which are related to specific disease features. The next step would be validating them in larger patients’ cohort but also, due to the specific and defined clinical characteristic these biomarkers are associated with, in other similar symptoms (and not only diseases). For example, biomarkers identified in DMD and associated with the presence of dilated cardiomyopathies, might be explored not only in rare diseases or NMDs but also in cardiac phenotypes; having SNPs identified as susceptibility to drug (corticosteroid) response, supports the rationale to search for these SNPs in other non muscle diseases in which therapy with corticosteroids gives different response, as autoimmunity or asthma. In this view, the impact of the BIO-NMD project is not only on NMDs but also on other disorders, which share with NMDs specific, fine tuned clinical features.

This previously unforeseen objective has higher impact as it can have a wider resonance in the human medicine and novel therapies. Apart from the results we have achieved in terms of quantity and quality of the biomarkers identified, the second added value of the project regards the approach we have followed (comparing patients’ categories with extremely well defined sub-phenotypes) and using (since we analyze a small number of samples) omic or wide targeted approaches. This combined and flexible strategy might be adopted for other similar rare diseases in order to address novel therapies, innovative diagnostics, and phenotype dissection.

Thirdly, for the technical innovation aspects, the BIO-NMD project has allowed all partners at implementing the knowledge on omic techniques, novel bioinformatics tools have been developed and can be used in a large variety of diseases, not only rare. The BIO-NMD database now contains data (SNPs, transcripts, proteins, and of course phenotypes) that can be used in other projects, and the recently approved EU Neuromics and RD-Connect projects will not only implement some aspects of the BIO-NMD but also benefit of the huge amount of data collected. Besides that, the availability of human samples, coming from various partners as well as from the Eurobiobank, also utilized within the BIO-NMD, have been finely characterized, categorized and disseminated among partners for being studied.

Project Context and Objectives:

Rapidly expanding knowledge of neuromuscular diseases (NMDs) has provided new targets for disease characterization, early diagnosis and drug development whilst presenting many challenges about how to translate this knowledge into clinical practice. This is all the more challenging as initial clinical trials typically run for such a short time that it is difficult for improvement to be effectively measured. Alongside this, NMDs are chronic degenerative diseases, often associated with severe muscle weakness making the characterization of clinical parameters challenging.

BIO-NMD project is a consortium of 12 partners coordinated by Prof. Alessandra Ferlini at the University of Ferrara. It is an EU-funded project devoted to the discovery and validation of biomarkers in muscle dystrophies with the aim of improving disease and therapy monitoring. It is a translational project focusing on Duchenne and Becker muscular dystrophies (DMD, BMD and other dystrophinopathies) and collagen VI-related myopathies (COL6 myopathies, which includes Ullrich congenital muscular dystrophy and Bethlem myopathy).

Biomarkers have been defined as cellular, biochemical, molecular alteration or biological characteristics that are measurable and evaluable in biological material as indicator of normal biological or pathogenic processes. Biomarkers may be used in, differential and early diagnosis, and in monitoring of disease progression, regression, or therapeutic. The project was privileged to use omic technologies (such as whole exome sequencing (WES) MaldiTof protein studies, multiplexes immunoassays) in order to maximize the discovery task. Targeted approaches such as SNP arrays, ELISA assays and antibody bead array have been used both for the discovery and the validation phases. Bioinformatics was crucial for

BIO-NMD was carried out both by developing novel tools for data analysis, and by dedicating software to WES analysis data storage, expression profiling and RNAseq analysis and variome interpretation. Thus, BIO-NMD contributed also to whole-OMIC
high throughput platforms development, application and optimization. Some customized/gene specific assays were also set up. WES analysis was performed in a group of DMD patients categorized depending on the disease severity (early/late loss of ambulation, normal/super survivors). These studies have identified several SNPs that modify the severity of the disease. These SNPs will be validated in larger cohorts and may also aid patient stratification in clinical trials, and perhaps lower the number of patients to be included in clinical trials.

Within the BIO-NMD project, partners have identified genetic modifiers that correlate with treatment response; in particular patients with high or low responsiveness to corticosteroid for DMD (SNPs) and cyclosporine A for COL6 patients (RNA profiling with up/down regulators). DMD carriers were investigated to discriminate symptomatic vs. non-symptomatic females based on SNPs search on a candidate gene list developed by a novel MedScan bioinformatics tool. This tool was also utilized in many steps of the project, generally for gene prioritization or for pre-selection of top genes which may act as modulators on the dystrophinopathies or COL6 myopathies.

Whole transcriptional studies allowed us to identify several nuclear long non-coding RNA and to demonstrate that they play a role in regulating dystrophin transcript abundance in skeletal muscle. Dystrophin transcription regulation was finely dissected using a novel customized ChIP on chiparray used to study DNA-protein interactions within the muscle cellular context. This approach unravelled novel transcription start sites, novel promoters region and novel splicing co-transcriptionally regulated mechanisms. Defining the regulation of dystrophin transcript will be deeply helping the optimization of the novel molecular therapies now ongoing for dystrophinopathies.

At the proteomic level the ideal biomarker has to be non/low-invasive. Therefore, research in BIO-NMD focused on the detection of muscle-derived proteins in body-fluids (serum and plasma) of DMD/COL6 patients. Within NMDs we used for the first time proteomics methods to validate protein markers for muscle deterioration in body fluids such as serum and plasma. Rapid technological development in combination with the increasing availability of large repository of antibodies has empowered antibody-based proteomics methods. One of the immunoassay technologies that has benefited is the suspension bead array platform. This platform offers a fast method for simultaneous analysis of hundreds of proteins in body fluids requiring only microliter amounts of samples. Using antibodies from the Human Protein Atlas program in combination with the suspension bead array platforms biomarkers identified in the project have been validated and their association with relevant clinical parameters was assessed. MedScan Software was used for gene prioritization and to identify top candidate biomarkers.

This approach allowed us to identify a few proteins that correlate with some specific clinical features of DMD. These data will be further biologically validated in larger cohorts or different NMD before entering into clinical practice. Regarding other sophisticated techniques to detect serum proteomic, as liquid chromatography coupled to an ion trap (LC-MS/MS) and an FTICRMS system, we detected a large variety of deregulated proteins. These have been prioritised based on function and on multiple evidences and a few of them have been explored using ELISA assay.

Moreover, blood levels of candidate proteins biomarker have been linked to clinical parameters (age at loss of ambulation, disease severity). Three proteins implicated in inflammation, extracellular matrix degradation and tissue fibrosis were selected for validation with the more established ELISA method. We demonstrated that serum levels of MMP-9 were correlated to disease progression in DMD patients compared to healthy controls.

Proteomic studies have been also performed in tissues/cells from both patients and murine models. In mice, relevant genes belonging to the autophagic and proinflammatory circuits have been demonstrated as markers of COL6 disorders. 'D-DIGE studies on COL6 and DMD muscles revealed an interesting scenario of up/down regulated proteins that clarified the etiopathogenesis of muscle pathology especially on COL6 myopathy. Many different proteomics screening pipelines have been therefore put in operation.

These pipelines have delivered important candidates for further validation studies. Since the throughput of the screening
pipelines is still relatively low and the quantification routines have limitations, confirmation and validation studies are important to extend the observation to larger patient cohorts. In a number of instances, biomarkers could be validated in larger cohorts. Bioinformatic tools have been also used (in addition to gene prioritization) to place the novel biomarkers on a specific cellular- biochemical context through the development of pathways that may be useful for potential therapeutic targets.

European Medicine Agency (EMA) regulatory aspects regarding BMs qualification processes have also been considered: specific documents and guidelines have been studied, illustrated and disseminated to the Partners to highlight the criteria to consider in the prospective of a future qualification application. Formal and informal contacts with EMA Innovation Task Force have also been held.

The BIO-NMD project was a three years project. To keep collaborations ongoing and to maximize the impact of our results three main actions were planned:

1) continuing collaborations via other projects both EU and from other funding agencies;
2) organizing a BIO-NMD workshop via the European NeuroMuscular Center (ENMC);
3) dialoguing via structured initiatives (briefing meetings, others) with EU authorities, especially EMA (Innovation Task Force), in order to disseminate results and to proceed in validating biomarkers, being therefore translated into clinical practice.

These are planned to be carried out in tight synergy with Industries, both those who have followed our project during these years and others that can be recruited via novel studies on other commoner diseases. Finally, the BIO-NMD network represents an excellence in Europe and might be therefore attractive for other non-EU collaborations that can reinforce the cooperative spirit of the EU research plans and strategies.

Project Results:

1) Patients' material organisation, circulation and managing of novel patients' fluids collection

WP1 has been responsible for ascertaining the availability of sufficient, high-quality, human biomaterials for the core research activities (genomics, transcriptomics, proteomics) of the BIO-NMD network. As work package leader, UNEW have identified and circulated a large number of samples relevant to the BIO-NMD project through close collaboration with partners and EuroBioBank. The BIO-NMD partners agreed on the need to adhere to the same patient classification proposed by the clinical working group.

2) Genomic biomarkers discovery by genome-wide analyses of DNA and RNA

One important step concerning this WP is the validation of the specificity of possibly identified biomarkers as modifiers in DMD and COL6 myopathies. The possibly identified biomarkers will therefore be screened in unrelated myopathy: dysferlinopathies, caused by mutations in DYSF gene, causing a wide spectrum of phenotypes mainly distal Miyoshi myopathy and LGMD2B.

Validation of the specificity of possibly identified biomarkers as modifiers in DMD and COL6 myopathies

CNVs as possible specific modifiers in DMD and COL6 myopathy:

The initial objective was to consolidate CNV data obtained in DMD and COL6 patients looking for these CNV in patients with unrelated myopathies (dysferlin protein deficiency). 8 patient samples have been analysed by CGH array.

We selected the NMD-CHIP WP2 design (for the analysis of genes known to be implicated in myopathies) instead of the initially
planned whole-genome CNV approach to obtain sufficient resolution for the detection of CNVs of small size. Several CNVs were identified in these samples. Comparisons could not yet be carried out as the CNV data obtained for DMD and COL6 patients within the timeframe of this project did not identify CNVs as candidate biomarkers.

SNPs as possible specific modifiers in DMD and COL6 myopathies

Another initial objective was to consolidate SNP data obtained in DMD and COL6 patients, looking for these SNPs in patients with unrelated myopathies (dysferlin protein deficiency).

We performed whole exome sequencing (Agilent exome capture and Illumina sequencing) on 37 samples. All SNP data have been grouped into 2 lists:
- all SNPs identified in those samples
- filtered data: all SNPs filtered through SNPdb and through the World Muscle Society Gene Table

SNP data from these experiments are now available for future comparison of SNP data from patients with DMD and COL6 myopathies.

Whole exome sequencing of DMD patients with varying severity

In order to find candidate biomarkers for DMD, we performed whole exome sequencing in two groups of DMD patients. In the first group the disease severity was defined by the age of loss of ambulation in patients on steroid treatment. Patients who lost the ability to walk before the age of 8.5 years were considered as early loss of ambulation and patients with loss of ambulation after 12 years of age were considered as late loss of ambulation.

DMD with long survival and DMD with early death or cardiomyopathy

All available DNA samples from DMD patients with long survival (alive after the age of 28 years) and DMD patients with early death (before the age of 18 years) or cardiomyopathy were tested. Eleven DMD patients with long survival and 7 DMD patients with early death/cardiomyopathy passed quality control and were processed for sequencing.

mRNAseq in a Symptomatic DMD female Carrier

We analyzed a female clinically evaluated as a severely affected symptomatic DMD carrier. Carrier status was confirmed by evidence of mosaic pattern of dystrophin expression on muscle biopsy; nevertheless, extensive analysis by MLPA, sequencing, CGH-array and RNA profiling failed to identify any mutation within DMD gene. We performed Whole Exome Sequencing in order to identify genetic modifiers that could explain symptomatic phenotype in this female.

ncRNAs within the DMD gene

We identified 11 new ncRNAs (9 sense oriented and 2 antisense oriented), 6 of them validated through Northern blotting and fully characterized by RACE PCR and sequencing. To understand whether the ncRNA expression might be functionally associated with that of dystrophin, we compared the expression of ncRNAs and dystrophin isoforms in six different cell lines. Expression of the ncRNAs is several orders of magnitude higher in cells displaying good levels of full-length dystrophin isoforms than in the other cell lines, thereby suggesting that ncRNAs and dystrophin expression follow similar dynamics.

The IncRNA expression resulted to be important for the transcriptional architecture of the DMD locus, since they regulate the dystrophin muscle and brain isoforms amount. We concluded that IncRNAs control muscle and brain dystrophin isoforms by
down-modulating their transcription level. In addition we found an inverse correlation between ncRNA expression and Dystrophin expression existed thus supporting the model of a negative regulation of IncRNAs on specific muscle dystrophin transcripts.

mRNAseq analysis in Col6 patients treated with CsA

We obtained muscle biopsies from three patients (one UCMD, a dominant BM and a recessive BM described in Merlini et al. PNAS 2008) before and after treatment with CsA (one month of oral CsA administration 5mg/kg) and performed mRNAseq analysis.

mRNAseq analysis in Col6 vs controls

We obtained muscle biopsies from three COL6 patients (one UCMD, a dominant BM and a recessive BM described in Merlini et al. PNAS 2008) and one control, and performed mRNAseq analysis on all of them. After the RNA-Seq reads were quality filtered and processed we used Cuffdiff to perform three pairwise comparisons of expression, splicing and promoter use between COL6 and control. By this approach the output data were then analyzed by Ariadne comparing COL6 patients with control allowing to drive the identification of many functional pathways and genes differentially expressed. Intriguing link with circadian rhythm genes have also been confirmed in mouse. We validated three out of four transcripts on other COL6 patients' biopsies (5 UCMD and 3 BM) and on other muscular pathologies (1 Dysferlin, 3 CAPN3, 2 myofibrillar) to confirm the circadian gene's differential expression and to check if they might be involved even in other muscular dystrophies.

Targeted resequencing and RNAseq analyses in DMD patients treated with steroids and different response

With concern to DMD patients with different response to corticosteroids, we collected 10 biopsies obtained from UNEW, UCL and UNIFE (5 patients with low response and 5 patients with high response to steroids). An mRNAseq experiment has been recently performed in two patients with low response to steroids and four patients with high response to steroids and data analysis is ongoing; discriminant analysis (DA) is used to determine which variables discriminate between two (or more) naturally occurring groups.

In order to consolidate the data obtained, UNIFE collected five more DNA of low responders DMD patients (UNEW) and at least 20 DNAs from BMD, DMD with mental retardation, UCMD and BM, Cystic Fibrosis and Beta Thalassemia patients each to be further sequenced on SOLiD. These DNA (a total of novel 86 samples) are currently under processing on SOLiD to technically consolidate the 34 SNPs already identified.

1628 genes were found, more or less differentially expressed in high steroid responders vs. low responders. We've compared this list with top 431 consistent genes from meta-analysis of several publicly available datasets, differentially expressed in DMD comparing to control normal patients. Overall, 39 intersected genes were found. Those genes, that have differently directed expression changes in DMD vs. normal tissues and high vs. low steroid responders, can be considered as promising candidates for being biomarkers of steroid response.

Targeted Resequencing of the Ariadne candidate genes identified 34 SNPs in 24 different genes significantly different in the two groups of patients and possibly explaining the different response to the steroid administration. SNPs will be further validated on the entire BIO-NMD consortium DNA repository (about 500 DNAs) for confirming their biological meaning as pharmacogenetic biomarkers predicting the steroid response in DMD.

DMD patients' myoblasts treated with AON and steroids:

In order to evaluate the influence of the glucocorticoid alpha-methyl prednisolone (PDN) on AONs treatment response, we
intend to use a cell-based assay with differentiated human myotubes, derived from DMD patients' biopsies. We will compare specific exon skipping efficiency in cell cultures treated/not-treated with PDN. We were also able to demonstrate that PDN positively influence exon skipping in treated cells, supporting a synergic effect on therapy.

We analysed by means of a new design of Fluidic card able to detect the DMD isoforms and the lncRNAs identified in the DMD gene (reported in Bovolenta et al. Plos ONE 2012) myoblasts from patients eligible for the treatment with AONs able to skip exon 51 in order to find a correlation between the expression of the ncRNAs and the restoration of the DMD gene reading frame.

We explored all transcripts originating from the DMD locus, including the IncRNAs identified by our array as well as the whole exome of the dystrophin gene and used the FluiDMD-RNA cards to detect reciprocal variations of dystrophin transcript (exome) and ncRNAs levels in four DMD patients before and after antisense oligonucleotides treatment.

We demonstrated that AON treatment rescue the physiological level on IncRNAs in cells, suggesting their role a transcriptomic biomarkers for AON therapy.

Candidate genes capturing system design:

Two alternative workflows for SNP and CNV detection in enriched exons (targets) were developed and used on the 5500xl SOLiD™ Sequencer and the Ion PGMTM Sequencer (Life Technologies) for Next Generation Sequencing. For the SOLiD™ System we used a probe capture bases enrichment of a total target of approximately 580kb. For the Ion PGMTM Sequencer we used a multiplex PCR based enrichment of approximately 150 regions to validate variants from WES. The significant reduction of target size (compared to Whole Genome and even Whole Exome sequencing) leads to a drastically increased sequencing depth of the target regions enabling higher variant calling and CNV accuracy. Further advantages are the option to multiplex using sample barcoding and reduction of sequencing cost.

FluiDMD cards

We specifically designed this platform to simultaneously detect del/dup mutations affecting the exon composition/sequence (small mutations, any types) as well as expression of the DMD gene isoforms. The FluiDMD was also found to be able to detect the decay of the DMD gene transcript induced by frame-shifting and/or stop mutations.

This suggests that the array could be useful as a very accurate technique for large-scale molecular diagnosis of DMD. For these reasons, we propose that FluiDMD could be used as a first-step screening method in the diagnostic procedure for DMD, if RNA is available, to be followed by MLPA and sequencing.

Developing tools (ChIp-on-chip) for defining the transcription dynamics of the DMD locus aiming at identifying cis and trans elements crucial to dystrophin transcription and splicing regulation

One intriguing aspect regarding the transcription of the DMD locus is that due to the gene size, RNA polymerase It takes about sixteen hours to complete synthesis of the primary transcript. How the polymerase can accomplish such a tremendous task is still completely unknown.

The complexity of the DMD locus is further increased by recent observations that the dystrophin region may also encode for a large number of long non-coding RNA whose function is still to be determined. We have approached these questions starting from a relatively simple issue: how is RNApol II distributed along the DMD locus and particularly which functional forms of polymerase are associated with the locus?
Our results suggest that some introns are marked by histone modifications typical of regulatory cis elements. We also concluded that these regions can exert a double and opposite activities on DMDs promoters: they repress the DMD brain promoter while activate DMD muscle promoter. These data strongly suggest that the identified regions may play a strategic role during the differentiation process activating the DMDm promoter and inhibiting all the others.

Developing novel low-invasive methods alternative to muscle biopsies in collagen VI myopathies and dystrophinopathies

Collagen VI expressing cells. Skeletal muscle biopsy is a choice method to monitor disease progression and the effects of treatments; however, it is an invasive and painful method and requires surgical expertise. Cultured fibroblasts are not useful for functional studies since they do not display the characteristic mitochondria alterations.

We evaluated the ability of hair follicle (HFc), adipose tissue (ATc) and circulating blood monocytes (MOMC) obtained from healthy subjects to proliferate, to be expanded and to synthesize collagen VI. By immunofluorescence analysis we found that all cell cultures synthesized and secreted collagen VI classical chains (alpha1-2-3 form); the alpha6 chain was only detected in cultures of HBc while the alpha5 chain was absent in all cell cultures. These data have been recently published (Gualandi, Muscle and Nerve 2011).

We also studied melanocytes from a UCMD patient carrying a homozygous mutation in COL6A2 gene. Our data indicate that melanocytes represent a promising cellular model to be used as biomarker for monitoring collagen VI related myopathies.

Characterization of novel collagen VI expression in human skin and muscle

Our study suggests that epidermal dystrophin acts in stabilizing melanocytes adhesion to the BM and that this function is impaired in DMD patients. Considering that melanocytes cultures can be easily obtained by conventional skin biopsies, they may represent a feasible and reliable cellular model for studying and monitoring dystrophinopathies.

We explored the mitochondrial phenotype in melanocytes of two DMD patients. By ultrastructural analysis, DMD melanocytes displayed morphological alterations of mitochondria which are very similar to that detected in mitochondria of muscle cells of the same patient.

3) Proteomic biomarkers discovery by studies on patient cells, muscle tissues and body fluids

In search of proteomic biomarkers, we followed a number of different parallel and mainly complementary strategies, maximizing our potential for protein biomarker discovery. Proteomics analysis of muscle biopsies focused on the identification of the different molecular pathways affected in muscular dystrophies. 2D-DIGE (two dimensional differential gel electrophoresis) followed by MS-based identification of differentially expressed proteins was the preferred method applied in this analysis.

We studied the differences between patients with severe and mild phenotypes (DMD vs. BMD) to better understand the complex pathophysiology and to define potential pharmacological targets and new biomarkers that may reflect the response to therapy. The latter is specifically relevant since the exon skipping therapy is at the most advanced stage of clinical trials and will potentially turn the Duchenne phenotype into the milder Becker phenotype.

Proteins with significant differences between DMD and BMD muscles were classified into 5 major categories:
- muscle contraction,
- muscle development,
- cytoskeletal rearrangement,
- metabolism,
Importantly, fibrinogen gamma was increased, suggesting a relationship between fibrinogen and increased fibrosis in DMD patients. Fibrinogen, in fact, is a soluble acute phase protein, which is released into the blood in response to stress. Apart from its key role in controlling blood loss following vascular injury, fibrinogen is also present at sites of inflammation and can increase the vascular permeability where it is immobilized and/or converted to fibrin. Fibrinogen was also identified in the serum-based studies mentioned below.

We further compared the muscle proteome of ambulant and non-ambulant DMD patients. There was considerable overlap between this and the previous, suggesting that biomarkers found in the DMD vs. BMD comparison are indeed biomarkers for disease progression.

Another study evaluated the difference between DMD patients with and without steroid treatment. Results suggest that steroid treatment mitigates the inflammatory response and contributes to the maintenance of muscle mass in non-ambulant subjects, but does not mitigate metabolic defects.

Despite its highly useful insights into the diversity of molecular mechanisms underlying muscular dystrophies, less invasive methods for biomarker detection would be preferred. In a number of studies, the serum proteome was studied as an alternative source for biomarker discovery. Proteomics on serum samples is challenging given the extremely large dynamic range of protein concentrations and the fact that the most interesting peptides are proteins are likely the medium to low abundant ones. Prefractionation of the serum proteome is therefore essential.

We conclude that pre-fractionation is essential for biomarker identification in the serum, but that even the extensive pre-fractionation schemes applied here are not sufficient and that more targeted approaches such as Multiple Reaction Monitoring (MRM) may be required for future diagnostic use.

Nevertheless, all approaches applied yielded relevant lists of candidate protein biomarkers for disease progression and response to therapy. These biomarkers have been further prioritized using the methods described in WP6 and are currently being evaluated in larger patient cohorts and longitudinal studies.

4) Exploratory biomarkers validation in humans

The workpackage 4 focused on the validation of candidate biomarkers discovered in WP2 and WP3 at the DNA, RNA and protein level.

A) DNA biomarkers

One of the major tasks within BIO-NMD was the identification and validation of genomic variants able to modify disease progression or response to treatment. We validated a previously described genetic modifier of DMD (SNP rs28357094 in the SPP1 gene) in 363 DMD patients from five different cohorts, with age of loss of ambulation as a read-out for disease severity. Surprisingly the T allele, which is associated with delayed loss of ambulation in the literature, showed a trend towards earlier loss of ambulation in our study. This trend was significant in one of the cohorts. Corticosteroid treatment appeared to delay the loss of ambulation by approximately two years. Significant differences in steroid treatment regimens may be partly responsible for the observed differences between genetic associations in different patient cohorts.

Eighty-four (84) potentially disease modifying SNPs identified by WP2 entered the validation phase. The SNPs included were identified in the female DMD-like WES study (see WP2) and in the study comparing DMD patients with early and late loss of ambulation. We observed only one significant association using the recessive model when corticosteroid
use was considered in the analysis. We conclude that more patient DNAs are needed to obtain sufficient power for detection of disease modifiers and that more refined clinical parameters should be included in genetic association analysis in rare (neuromuscular) diseases.

**B) RNA biomarkers**

We have worked on the identification of new diagnostic biomarkers for DMD. The newly developed Flui-DMD assay is able to identify almost all out-of-frame mutations in the DMD gene at the RNA level using the observation that the decay rate of the DMD transcript accelerates after the mutation site. We further focused on the validation of previously published muscle-specific miRNA biomarkers in the serum.

A significant increase in the level of two of the analysed miRs was seen in the serum of DMD and BMD patients compared to healthy controls. One of the significantly increased miR showed also statistical significance when its level was compared in non-ambulant and ambulant DMD patients (p=0.005) suggesting that the miR may represent a valuable biomarker for DMD disease progression. More in-depth analysis are ongoing. Moreover, serum miRNA levels have been measured in clinical trial protocols with dystrophin restoration therapies.

**C) Protein biomarkers**

ELISA was used to validate single protein biomarkers in the serum and plasma of large cohorts of DMD, BMD and COLVI patients. TIMP-1, MMP-9 and FN demonstrated consistently higher levels in DMD and BMD patients over controls. Of these, MMP-9 is the most promising biomarker for disease progression based on analysis of more than 300 independent patients and longitudinal analysis (with one year time intervals) in two well characterized DMD patients cohorts. Serum MMP-9 levels were also elevated in patients with Bethlem and Ulrich myopathy compared to controls. However, the raise in the MMP-9 levels was less pronounced than for DMD/BMD. Still, the usefulness of MMP-9 as biomarker for muscular dystrophies other than DMD should be carefully considered.

The antibody suspension bead array is the preferred high-throughput platform for protein biomarker validation in plasma or serum samples. This platform was used to screen >300 candidate protein biomarkers identified based on the meta-analysis (WP6) of high-throughput data from multiple studies performed in WP2 and WP3.

Given inter-cohort differences, data were first analyzed within cohorts. Subsequently, proteins identified in multiple independent comparisons were regarded as most promising biomarkers for DMD and BMD respectively. Among the most significant protein markers we identified proteins associated with muscle function, indicating that muscle proteins are leaking from skeletal or cardiac muscle due to sarcolemmal. This opens the possibility of using blood as an indicator for muscle wasting and disease progression. Three mitochondrial proteins are novel protein biomarkers that have not been previously reported to be associated with NMD. Three mitochondrial proteins have also been identified to be associated with DMD and BMD. The abundance of these markers is higher in DMD than in BMD and lower in control serum or plasma in comparison to BMD. Further validation of these proteins as biomarkers for disease progression and their association with disease phenotypes needs to be performed in longitudinal studies and additional patient samples.

5) Biomarkers discovery and validation on animal models

The WP5 activities concentrated on studies aimed at obtaining novel information on the pathogenic routes and the pathophysiological mechanisms leading to the dystrophic phenotype, with the aim of identifying new candidate biomarkers to be translated in patients. Based on the known data about the structural and functional defects of COL6 diseases and DMD, WP5 carried a number of experiments in Col6a1 null and mdx mice in order to explore novel hypotheses on the cellular and
molecular mechanisms underlying muscle pathology. These experiments brought to the discovery of new pathway involved muscle pathogenesis, and most efforts were spent on the thorough analysis and understanding of such pathways. The work carried out by WP5 partners identified key molecules that are deregulated in these models for MDs and disclosed novel targets for therapy, also suggesting possible treatments acting specifically on these molecules. Moreover, the studies performed by WP5 also disclosed some biomarkers that are sensitive to pharmacological treatments.

The studies carried out by WP5 analysed the muscles of the animal models at the molecular and cellular levels, in physiological conditions and after diverse treatments, using drugs or challenging the mice with different stresses. Whole muscles were dissected from the mice, but also myofibers and primary muscle cell cultures were obtained from the mutant mice. Molecular studies were made at the RNA (RT-PCR, RNA seq) and protein (western blot, proteomic analyses, immunohistochemistry) levels. The results obtained by the different omic approaches were then analysed in order to evaluate the role of each putative biomarker by specific programs developed within the project (Medscan-NMD and Pathway Studio-NMD).

6) Bioinformatics tools for identifying functional pathways, potential targets and data outflow integration

Design of a friendly database and data compilation

We combined data from various projects including databases developed in the FP6 TREAT-NMD Project (Translational Research in Europe - Assessment and Treatment of Neuromuscular Diseases) and the FP7 NMD-CHIP Project (Development of targeted DNA-Chips for High Throughput Diagnosis of Neuro Muscular Disorders) as well as data from various Locus Specific DataBases using the UMD reference software (UMD-LSDBs). We created the BIO-NMD database using the 4th Dimension Relational Database Management System (RDMS), accessible directly from the Internet using a secured transfer protocol (https) as well as logins and passwords.

Design and use of a high throughput process to analyse genomic data

We developed a new system called UMD-HTS (UMD High Throughput Sequencing) that can handle the large sets of data generated by NGS and perform pathogenicity predictions for each SNP (Single Nucleotide Polymorphism). This tool will be used both by BIO-NMD partners and will also be made available to partners from the NMD-CHIP EU project. In order to perform predictions using the UMD-Predictor algorithm, data were collected at various levels including conservation, physicochemical properties and impact on splicing signals. This led to a total of 3,957,224,568 annotations. Various technical solutions were evaluated in order to design the most efficient solution (database structure, parallel computing). The final solution contains 24 satellite databases (one per chromosome) and a central system known as the UMD-HTS system. In addition, the UMD-HTS system is one of the first systems able to predict the potential activation of cryptic acceptor/donor splice sites by deep intronic mutations (69 out of 1189 deep intronic mutations).

In silico identification and validation of biomarkers from NMD-related pathways analysis

ARIADNE created a knowledge-rich environment dedicated to the search of NMD-related genes (BIO-NMD knowledgebase). ARIADNE used a customized version of MedScan (BIO-NMD MedScan cartridge), its proprietary text mining tool, to automatically extract molecular interactions from public scientific literature sources. Additional manually curated contents, as NMD-model pathways and experimental data analysis, were also included in the BIO-NMD knowledgebase. ARIADNE scientists built model pathways related to NMD diseases and performed meta-analysis of microarray GEO datasets. ARIADNE MedScan technology was customized to build a literature-derived biological knowledge base focusing on neuromuscular diseases. 70 concepts and 600 Aliases/Terms related to neuromuscular diseases have been added for improving extraction capability of relevant information related to NMD. All PubMed abstracts as well as all free full text articles
as of May 2010 were extracted using the custom MedScan NMD cartridge to populate the Bio-NMD knowledgebase with >1.6M relationships between different entity types (proteins, genes, diseases, cell processes, small molecules, etc). Among them, a total of 887 entities were identified as being of interest for neuromuscular diseases and 387,212 relations involving these entities were extracted.

The BIO-NMD knowledgebase prototype is composed of 3 major sources of information: biological relationships extracted by the MedScan-NMD prototype and supported by literature, hand-curated model pathways for NMD diseases built by ARIADNE scientists, experimental datasets and biomarker candidate lists from public or partners’ sources.

One critical constituent of the analysis workflow for biomarker discovery is the Sub-network enrichment analysis (SNEA) developed by Ariadne and integrated to Pathway Studio®. SNEA is a very powerful algorithm for functional analysis of high-throughput data on the level of potential regulators. This tool is a variation of the Gene Set Enrichment Analysis (GSEA) algorithm. Unlike conventional GSEA which uses predefined collection of gene sets, SNEA uses the entire literature-extracted global protein-protein expression network - here from the Bio-NMD knowledgebase - to generate a comprehensive collection of gene sets, each representing immediate downstream expression targets of each individual protein (‘seed’) in the global expression network. The central idea of SNEA approach is that if the downstream expression targets of the ‘seed’ protein are enriched with differentially expressed genes then the ‘seed’ protein is one of the key regulators of the differential expression profile. The SNEA is also of high value for considering experimental data when building mechanistic model pathways.

Conclusion

- The UMD-HTS system to analyse data from NGS platforms has been developed. It is the first of its kind as it can from full exome or transcriptome sequencing experiments:
  o Name mutations using the HGVS international nomenclature system;
  o Search for potential misalignment using BLAT;
  o Search for variation description including variation frequency in dbSNP;
  o Perform pathogenicity prediction using UMD-Predictor algorithm and
  o Predict the potential impact on splice sites of exonic and intronic mutations.
- The BIO-NMD database prototype has been developed. It includes all relevant information for the BIO-NMD project identified by all partners. It is a relational database, developed with the 4th Dimension Relational Database Management System (RDMS) designed to be accessible directly from the Internet using a secured transfer protocol (https) as well as logins and passwords.
- Major developments have led to the release of the Bio-NMD knowledgebase, which is constituted of 3 major sources of information: biological relationships extracted by the MedScan-NMD prototype and supported by literature; hand-curated model pathways for NMD diseases built by Ariadne’s scientists; and experimental datasets and biomarker candidate lists from public or partners’ sources. The Bio-NMD knowledgebase is fully integrated with the existing software architecture developed by Ariadne called Pathway Studio®. It is an open-platform designed to continually integrate additional contents and to import data generated by partners including output data from the other bioinformatics resources (UMD-HTS, BIO-NMD database). The integrative platform, especially the knowledge-rich environment developed by ARIADNE helps to capture neuromuscular disease mechanisms and facilitates translational research activities for the identification, prioritization and validation of the different types of candidate biomarkers from disease diagnostic biomarkers to treatment efficacy biomarkers for personalized medicine.

7) Biomarkers regulation and qualification at the EMEA

The major objectives of WP7 were:
1) Monitoring the data that the different partners produced during the project regarding new BMs, in order to guide them in the knowledge of EMA rules and guidelines in the prospective of a future BMs qualification procedure
2) Contacts with the regulatory agency (mails, calls, Teleconferences) in order to apply for a Briefing Meeting or Qualification
Procedures regarding some newly identified and validated BMs in the context of the Consortium.

UNIROMA, during the course of the project, particularly after the first year, has continuously followed and monitored the data that all partners of the BIO-NMD project have presented in the various consortium meetings, addressing the partners in the understanding of EMA guidelines about data collecting and about procedures for the possible future submission of these results, for consequent eventual request of an EMA Briefing Meeting or Scientific Advice or for a future biomarker qualification application.

At this aim the role of WP7 has been to study, explain and disseminate to the Partners the EMA regulatory Guidelines and documents or EMA Concept Papers related to BMs qualification, with particular regards to Genomic BMs (GBM). In particular the criteria has been illustrated and highlighted, important to consider for some of the BIO-NMD partners who are working on Genomic BMs also related to Drug response.

UNIROMA, in order to circulate this information, and also with the collaboration of UNEW, has also uploaded in BIO-NMD intranet website (see http://intranet.treat-nmd.eu online) all these EMA Documents.

Different issues have been faced regarding Biomarker regulatory aspects and have been discussed with other BIO-NMD partners both at the BIO-NMD meetings, both by informal contacts with interested partners or by Teleconferences. In fact, after having illustrated the different EMA guidelines regarding these aspects, different issues have been faced on the possible impact or implications for the BIO-NMD consortium and NMDs.

During the seven last months, in light of the progress of the different partners work on the new BMs identification and validation, and in light of the illustration of some interesting preliminary results on some prognostic and/or pharmacogenomics BMs (both genomics or proteomics ones), UNIROMA has illustrated and explained the possibility to ask a 'Briefing Meeting' to EMA 'Innovation Task Force' to illustrate these preliminary results and to have an informal discussion and data sharing in order to discuss the further steps to a possible future Qualification Process of some of these BMs. In fact, UNIROMA has explained that the objective of these Briefing Meetings is to facilitate an informal process of sharing scientific and technical information between applicants and regulators, to allow in an informal setting scientific and regulatory issues that arise by the inclusion of new BMs/methodologies, in the development strategy and to assess their potential implications in the regulatory processes.

A discussion on these issues has been developed among different partners also at the Stockholm (MAY 2012) and Rome (November 2012) BIO-NMD meetings. UNIROMA has also advised other Partners that this kind of meeting could be the right one for the kind of work carried out by the Consortium and that these Briefing meetings can be asked to 'Innovation Task Force' (ITF) before to ask for a 'Formal' Scientific Advice to the CHMP Scientific Advice working party.

It has been discussed that it would be important to have this kind of meeting with ITF also in order to discuss with them the problem arising by the fact that current EMA documents and guidelines regarding gBM are often focused on large population studies for multifactorial diseases and it would be very important to consider the different case of Prognostic or Pharmacogenomics gBMs in the field of rare diseases like NMDs studied by the consortium.

UNIROMA has studied the EMA ‘Standard Operating Procedures’ to ask the organization of a Briefing meeting and has then started the informal and formal Contacts with EMA Innovation Task Force Secretariat and Members. On 25th of October a TeleConference has been held regarding this Issue (see also deliverable D7.4) with 3 of these EMA ITF Members. Then, in the final BIO-NMD meeting in Rome, WP7 Leader has illustrated the results of this Teleconference. After an important discussion about this possibility of a Briefing Meeting with EMA as a Consortium, UNIROMA have sent to the Partners at the end of November 2012 a Questionnaire in order to clarify their interest and to ask them the principle BMs they think should be presented.
Finally, UNIROMA has participated to the preparation of a Manuscript on Ethic, Regulatory and Social Aspects of BMs, (in collaboration with UNEW and UNIFE and Ariadne): 'The importance of biomarkers in the field of rare neuromuscular disease: how current research and regulatory procedures may help translate progress into improved therapies' (Cathy Turner, Paola Borgiani, Michael Lebowitz, Cinzia Ciccacci, Hanns Lochmuller, Pauline McCormack, Elena Schwartz, Volker Straub, Simon Woods, Giuseppe Novelli, Alessandra Ferlini).

The general positive result is that all partners have had the possibility to know these very specific regulatory aspects to consider and to take into account in the prospective of the Briefing Meeting with EMA Innovation Task Force to present and to discuss the major results of the Consortium about identified and validated BMs, to share with them some particular issues related to BMs in rare NMDs. This step is particularly important in the prospective of a possible future qualification process.

The fact that, in only 3 years, the consortium, by means of WP7, has already reached the step of the contact with EMA to start the process of a possible new BM qualification, can be considered a good result.

Potential Impact:

Economic impacts

Novel drugs/therapies development

Over the last several years, pharmaceutical companies have shown significant interest in conducting groundbreaking research and development for effective treatment agents for NMD. Recently, the EMA developed a process that would make prevalence for rare disease products.

Results of BIO-NMD Consortium can make a significant progress in novel therapeutic development in rare disorders including NMD because of the identification of sensitive and reliable biomarkers that can be used for facilitating the evaluation of emerging therapies in drug trials and regulatory approval in NMD. The use of this biomarker's panel for the diagnosis, prognosis, and monitoring of NMD as well as to guide the choice of therapeutic regimens may significantly improve the current clinical practice. While the market for any one of these rare diseases is rather small, the aggregate world-wide market is predicted to exceed $500M per year. An industrial partner (for example, Ariadne-Dx, LLC) in addressing this niche market, expects to obtain certain rights to exclusivity and regulatory advantage due to the orphan status of these diseases.

Early diagnosis and screening

A longstanding debated question for rare disease is the feasibility/availability of neonatal tests (newborn screening). Having RDs diagnosed at birth will surely enhance their care, their monitoring and at the end will maximize the benefit of novel therapies. A precocious diagnosis is prerequisite for early care of these patients, which impact also on family psychological and social aspects. The huge effort the BIO-NMD has put in developing high throughput techniques will surely facilitate the establishing of the disease accurate diagnosis by validating some specific biomarkers in pivotal studies (as SNPs or serum biomarkers). This will have economical repercussion on disease diagnosis and will speed up access to both standardized and novel therapies.

Pharmacogenetics

EMA considers orphan drug a task with research priorities and dedicated regulatory modalities, considering the socio-economical impact rare diseases do have on the health care. During the drug development process, having a biomarker able to predict drug response and/or occurrence of side effects will be of outmost importance and will reduce the costs of the drug development. Within the BIO-NMD some SNPs related to drug (corticosteroid) responses emerged, and if they will be
biologically validated in larger cohort, could be qualified for screening DMD patients before undergoing to steroid therapies, reducing the risk of side effects and increasing efficacy of the treatment. Some SNPs and serum/plasma biomarkers (as MMP9) have been identified as associated with disease severity or specific disease characteristics. These biomarkers will serve for patients' stratification, for selection of appropriate subjects for clinical trials. This will impact on the economical load, patients' care and novel therapies do have, and having these costs reduced will allow wider patients participation to novel trials and encourage novel drug qualifications.

Bioinformatics

A bottleneck of rare disease is analyzing the huge amount of data generated by the high throughput techniques (whole exome sequencing transcriptome sequencing, proteomic profiling). The bio-informatics tasks in the BIO-NMD have addressed this issue by developing novel software (MedSCAN and PathwayStudio) to analyse data. In particular, the method revealed to be very effective to prioritize gene for further analysis and validation. This allows saving time and sparing resources in order to identify biomarkers.

Social and ethical aspects

BIO-NMD's primary aim was to discover biomarkers for neuromuscular disease - in particular dystrophinopathies and collagen VI disease. The significance of biomarker discovery for patients and their families is manifold. Part of the task for WP8 has been to communicate these benefits to the patient community. This has been achieved in part through the patient section of the BIO-NMD website which was also translated into German, French, Dutch and Italian. In addition, a poster was presented at patient organisations' conferences (Spierziektecongres Nederland, Veldhoven, 15 Sept 2012).

A major application of new biomarkers is in clinical trials. At the moment, when a new drug is being tested, researchers use a variety of ways to measure whether the drug has had a positive effect. However, these measures are not always very good at showing small changes and improvements in symptoms, especially when the drugs are only being tested for a short time. However, if the BIO-NMD project can find biomarkers in patients' blood or urine, samples of these can be taken throughout clinical trials. Measuring the levels of these biomarkers would show researchers clearly and accurately whether the drug being tested has had an effect or not.

Other benefits of biomarkers include:
- Blood and urine testing may be able to replace the use of muscle biopsies in the future
- Diagnosis can happen earlier because testing for biomarkers is quicker and easier than genetic testing
- Disease progression can be accurately measured allowing better clinical management of symptoms
- Existing treatments can be adjusted to precisely meet the needs of individual patients and ensure they get the maximum benefit.

A patient focussed newsletter has been produced every 9 months giving an explanation of the background of the project, including the importance of biobanking, a discussion about translational research and a report on the progress of BIO-NMD and its expected impact.

The project has presented some promising results. Several biomarkers identified during BIO-NMD are currently under further study in order to move towards their validation. WP9 (below) describes the presentation of these results in more detail. However, the potential impact of the foreground is significant. Should any of the biomarkers be validated, their use in clinical trials may speed the drug development pipeline by acting as a surrogate end point or by improving the stratification of patient cohorts. This may reduce the cost to the pharmaceutical industry and improve the chances of trial success. Validated biomarkers may be shown to be of use and significance for other neuromuscular diseases so widening the impact of BIO-NMD beyond the initial scope of the project.
The ethical implications of BIO-NMD have not been overly complex. The licences required were obtained at the outset and the PEB met every 6 months. Over the course of the project, the PEB was asked to consider what the approach should be to any incidental findings (IFs) and this prompted an interesting debate which involved partners, the PEB, the PAC and other patient representatives. It was agreed that IFs were very unlikely in the BIO-NMD project as the sequencing work was targeted. Indeed, no IFs arose.

Another area of discussion for the PEB and PAC was on informed consent by patients for the use of their samples. Again, in BIO-NMD, there were no problems and the approved forms in each centre were used when collecting samples for partners to study.

Exploitation and dissemination

Dissemination activities

One of the most important dissemination tools for BIO-NMD has been the project website at http://www.bio-nmd.eu was created in the first few months of the project. It gives:
- the aims and objectives of the research
- details about the project in lay terms, aimed at the patient community
- information about each partner and their role in BIO-NMD with links to their own websites
- details about the composition and roles of various committees including those which deal with IP which will be important during any exploitation activity
- a list of publications which have come out of the BIO-NMD project
- a detailed summary every 6 months of the progress made
- contact details for further information
- news and event details
- forms to facilitate partners submitting information about their dissemination activities.
- a recently added form for Industry representatives to express an interest in the M36 Industry session along with details of that session.

The website is well used with over 10,000 visitors to the site over the course of the project. These visitors viewed more than 36000 pages (data from Google analytics).

IPR management

Each partner has nominated a representative that will deal with all the IP aspects related to the BIO-NMD project within his/her organisation and will also participate in the periodic IPC meetings. A plan for the exploitation of Foreground was written by UNEW as a document for discussion. This was circulated to all partners in advance of the M18 meeting in Freiburg in order that the ideas in it could be discussed.

Patentability has been identified for some of the exploratory biomarkers (human) validated by BIO-NMD. The value of protecting the results has to be considered by partners before filing a patent application.

The TTO coordinator or a private company who has had a major contribution to the results (potential IP owner) and has expertise in the field will take over lead for filing patent.

Cooperation with industry

A first meeting with representatives from industry was held on 8th July 2010 in London, during the M6 Bio-NMD Steering
Committee. The aim of this session was to present to industry the objectives, structure and partners of the BIO-NMD project.

The ‘industry’ participants confirmed their interest in this kind of meeting which provided a good forum for sharing information and project results. This appeared as a good format for future interaction and collaboration between academic professionals and industry professionals who could then realise the potential opportunities to advance the basic science, translatability and ultimately patient treatment options. Validating a translatable biomarker of diagnosis, pathophysiology and treatment could be of interest for industry and would help them to search for alternative starting points for drug discovery programmes. A second industry meeting was organized during the final project meeting in Rome on 14th November 2012.

Partners of BIO-NMD consortium presented the work and the results achieved in the project and could answer to all the questions from Industry participants. The fruitful discussion between all the attendees highlighted first the interest of industry in the biomarkers discovered in BIO-NMD project, and secondly the importance of a future collaboration to assess how these biomarkers could be used. Indeed, a number of biomarkers have been generated by the project and their impact will not only be on the two main diseases object of the project, but also on many other neuromuscular diseases. Some biomarkers as SNPs related to drug response might be exploitable also in non-muscle disorders. Cooperation with industry is essential now to translate biomarkers in trials, and in clinical stratification.

Project website: http://www.bio-nmd.eu

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