TELETHON – APPLICATION GUP04008

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General Info

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Project Title

A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations

Telethon UILDM Grant number: GUP04008

Applicant name: Vincenzo Nigro

Centres involved in the study

(Coordinator + Partner/s)

1

Project duration

2 years

Type of application

Old Applicant – New Application

Clinical Research

Clinical Research – Genetic muscle and nerve disorders, and spinal muscular atrophy (SMA)

Disease name

MUSCULAR DYSTROPHY, DUCHENNE TYPE; DMD

OMIM Number (optional)

310200

Research type

Data banks and national networks
Development of new diagnostic methods
Diagnostic protocols

Do you have Ethical Committee approval?

Does your study involve vertebrate animals?

NO

Applicant – Personal data

Applicant – Personal data

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Personal data

Name

Vincenzo

Surname

Nigro

Gender

Male

Birthdate (dd/mm/yyyy)

28/07/1960

Place of birth

Napoli

Nationality

Italy

Address where correspondence should be sent

Department/University/Institution/Laboratory

Dipartimento di Patologia Generale/Seconda Università degli Studi di Napoli
Facolta di Medicina e Chirurgia

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Applicant – Curriculum Vitae

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Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Curriculum Vitae

Education and training

NAME Vincenzo Nigro, M.D. POSITION TITLE Associate Professor, II Univ. of Naples
INSTITUTION AND LOCATION DEGREE YEAR CONFERRED FIELD OF STUDY
University of Naples, Italy M.D. 1987 Medicine
Research and Professional Experience
1987–1988 Postdoctoral Fellow in General pathology at the Institute of General pathology, University of Naples, Italy;
Director: Prof. F. Bresciani.
1988–1991 Postdoctoral Fellow at the International Institute of Genetics and Biophysics; CNR, Naples, Supervisor: Prof. E. Boncinelli
1992–1999 Assistant Professor in General Pathology (F04A) at the Institute of General pathology, II Univ. of Naples
2000–Present Associate Professor in General Pathology (F04A) at the Institute of General pathology, II Univ. of Naples
Honors
1988–1990 A.I.R.C. (Italian Association for Cancer Research) fellowship.

Employment and Research Experience

Assistant professor since 1992 and associate professor (2000) of general pathology (MED/04). He is Professor of General Pathology at the School of Dentistry and at the Specialization Schools of Pediatrics, Clinical Pathology, Oncology, Internal Medicine, Endocrinology and Metabolism, Cardiosurgery, Medical Genetics and in the "Diplomi Universitari". He is member of the PhD board in Medical Genetics. He is at the Department of General pathology directed by F. Bresciani.
He begun in 1982 at the "Istituto di Patologia Generale e Oncologia" of the University of Napoli to work as a student under the supervision of G. A Puca and after the degree he got a fellowship of the Italian Cancer Association (AIRC) to study the mechanism of action of estrogen receptor. Since 1989 he worked at the International Institute of Genetics and Biophysics of CNR in the lab of E. Boncinelli and A. Simeone (developmental biology, identification of transcription factors required for the embryogenesis and the CNS development). In 1992 he constituted his own research team for the molecular genetics of muscular dystrophies. He published 60 full papers (10 as first author) in international journals (seven articles with i.f.>10, two with i.f.>30), some of reference in the specific field of muscular dystrophy, like the identification of delta−sarcoglycan and its mutations that cause limb−girdle muscular dystrophy (LGMD2F, 150 citations)) and the identification of the Syrian hamster cardiomyopathy gene, one of the principal animal models. He contributed to gene discovery in at least 20 different genetic diseases since he is coordinator of the mutation detection core facility" at the Telehon Institute of Naples (TIGEM), directed by A. Ballabio. He is principal investigator of TIGEM−University joint research projects on the identification of genes involved in muscular dystrophies.
Visit: www.vincenzonigro,it

Publications

1: Fanin M, Fulizio L, Nascimbeni AC, Spinazzi M, Piluso G, Ventriglia VM, Ruzza G, Siciliano G, Trevisan CP, Politano L, Nigro V, Angelini C.
Molecular diagnosis in LGMD2A: Mutation analysis or protein testing?
Hum Mutat. 2004 Jul;24(1):52−62.
PMID: 15221789
2: Nigro V.
Molecular bases of autosomal recessive limb−girdle muscular dystrophies.
Acta Myol. 2003 Sep;22(2):35−42. Review.
PMID: 14959561
3: de Paula F, Vieira N, Starling A, Yamamoto LU, Lima B, de Cassia Pavanello R, Vainzof M, Nigro V, Zatz M.
Asymptomatic carriers for homozygous novel mutations in the FKRP gene: the other end of the spectrum.
Eur J Hum Genet. 2003 Dec;11(12):923−30.
PMID: 14647208
4: Fischer D, Aurino S, Nigro V, Schroder R.
On symptomatic heterozygous alpha−sarcoglycan gene mutation carriers.
Ann Neurol. 2003 Nov;54(5):674−8.
PMID: 14595658
5: Politano L, Nigro G, Nigro V, Piluso G, Papparella S, Paciello O, Comi LI.

Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results.
Acta Myol. 2003 May;22(1):15–21.
PMID: 12966700
6: Tammaro A, Bracco A, Cozzolino S, Esposito M, Di Martino A, Savoia G, Zeuli L, Piluso G, Aurino S, Nigro V.
Scanning for mutations of the ryanodine receptor (RYR1) gene by denaturing HPLC: detection of three novel malignant hyperthermia alleles.
Clin Chem. 2003 May;49(5):761–8.
PMID: 12709367
7: Piluso G, Carella M, D'Avanzo M, Santinelli R, Carrano EM, D'Avanzo A, D'Adamo AP, Gasparini P, Nigro V.
Genetic heterogeneity of FG syndrome: a fourth locus (FGS4) maps to Xp11.4–p11.3 in an Italian family.
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PMID: 12522552
8: de Paula F, Vainzof M, Passos–Bueno MR, de Cassia M Pavanello R, Matioli SR, V B Anderson L, Nigro V, Zatz M.
Clinical variability in calpainopathy: what makes the difference?
Eur J Hum Genet. 2002 Dec;10(12):825–32.
PMID: 12461690
9: Nobile C, Toffolatti L, Rizzi F, Simionati B, Nigro V, Cardazzo B, Patarnello T, Valle G, Danieli GA.
Analysis of 22 deletion breakpoints in dystrophin intron 49.
Hum Genet. 2002 May;110(5):418–21.
PMID: 12073011
11: Ikeda Y, Gu Y, Iwanaga Y, Hoshijima M, Oh SS, Giordano FJ, Chen J, Nigro V, Peterson KL, Chien KR, Ross J Jr.
Restoration of deficient membrane proteins in the cardiomyopathic hamster by in vivo cardiac gene transfer.
Circulation. 2002 Jan 29;105(4):502–8.
PMID: 11815435
13: Dincer P, Bonnemann CG, Erdir Aker O, Akcoren Z, Nigro V, Kunkel LM, Topalolu H.
A homozygous nonsense mutation in delta–sarcoglycan exon 3 in a case of LGMD2F.
Neuromuscul Disord. 2000 Jun;10(4–5):247–50.
PMID: 10838250
14: Date M, Otsu K, Nishida K, Toyofuku T, Matsumura Y, Morita T, Hirotani S, Okazaki Y, Hayashizaki Y, Nigro V, Kuzuya T, Tada M, Hori M.
Single–strand conformation polymorphism analysis on the delta–sarcoglycan gene in Japanese patients with hypertrophic cardiomyopathy.
Am J Cardiol. 2000 Jun 1;85(11):1315–8.
PMID: 10831946
16: Piluso G, Mirabella M, Ricci E, Belsito A, Abbondanza C, Servidei S, Puca AA, Tonali P, Puca GA, Nigro V.
Gamma1– and gamma2–syntrophins, two novel dystrophin–binding proteins localized in neuronal cells.
J Biol Chem. 2000 May 26;275(21):15851–60.
PMID: 10747910
17: Abbondanza C, Medici N, Nigro V, Rossi V, Gallo L, Piluso G, Belsito A,

Roscigno A, Bontempo P, Puca AA, Molinari AM, Moncharmont B, Puca GA.
The retinoblastoma−interacting zinc−finger protein RIZ is a downstream effector of estrogen action.
Proc Natl Acad Sci U S A. 2000 Mar 28;97(7):3130−5.
PMID: 10706618
19: Sampaolo S, Puca AA, Nigro V, Cappa V, Sannino V, Sanges G, Bonavita V, Di Iorio G.
Lack of sodium channel mutation in an Italian family with paramyotonia congenita.
Neurology. 1999 Oct 22;53(7):1549−55.
PMID: 10534266
20: Rivier F, Robert A, Hugon G, Bonet−Kerrache A, Nigro V, Fehrentz JA, Martinez J, Mornet D.
Dystrophin and utrophin complexed with different associated proteins in cardiac Purkinje fibres.
Histochem J. 1999 Jul;31(7):425−32.
PMID: 10475570
22: Moreira ES, Vainzof M, Marie SK, Nigro V, Zatz M, Passos−Bueno MR.
A first missense mutation in the delta sarcoglycan gene associated with a severe phenotype and frequency of limb−girdle muscular dystrophy type 2F (LGMD2F) in Brazilian sarcoglycanopathies.
J Med Genet. 1998 Nov;35(11):951−3.
PMID: 9832045
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Exclusion of identified LGMD1 loci from four dominant limb−girdle muscular dystrophy families.
Hum Hered. 1998 Jul−Aug;48(4):179−84.
PMID: 9694248
29: Nigro V, Okazaki Y, Belsito A, Piluso G, Matsuda Y, Politano L, Nigro G, Ventura C, Abbondanza C, Molinari AM, Acampora D, Nishimura M, Hayashizaki Y, Puca GA.
Identification of the Syrian hamster cardiomyopathy gene.
Hum Mol Genet. 1997 Apr;6(4):601−7.
33: Nigro V, de Sa Moreira E, Piluso G, Vainzof M, Belsito A, Politano L, Puca AA, Passos−Bueno MR, Zatz M.
Autosomal recessive limb−girdle muscular dystrophy, LGMD2F, is caused by a mutation in the delta−sarcoglycan gene.
Nat Genet. 1996 Oct;14(2):195−8.
PMID: 8841194
34: Nigro V, Piluso G, Belsito A, Politano L, Puca AA, Papparella S, Rossi E, Viglietto G, Esposito MG, Abbondanza C, Medici N, Molinari AM, Nigro G, Puca GA.
Identification of a novel sarcoglycan gene at 5q33 encoding a sarcolemmal 35 kDa glycoprotein.
Hum Mol Genet. 1996 Aug;5(8):1179−86.
35: Politano L, Nigro V, Nigro G, Petretta VR, Passamano L, Papparella S, Di Somma S, Comi LI.
Development of cardiomyopathy in female carriers of Duchenne and Becker muscular dystrophies.

JAMA. 1996 May 1;275(17):1335–8.
PMID: 8614119
41: Nigro V, Nigro G, Esposito MG, Comi LI, Molinari AM, Puca GA, Politano L.
Novel small mutations along the DMD/BMD gene associated with different phenotypes.
Hum Mol Genet. 1994 Oct;3(10):1907–8.
PMID: 7849724
47: Nigro V, Politano L, Nigro G, Romano SC, Molinari AM, Puca GA.
Detection of a nonsense mutation in the dystrophin gene by multiple SSCP.
Hum Mol Genet. 1992 Oct;1(7):517–20.

Host Institution

Host Institution

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Host Institution

Department/University/Institution

Department of General Pathology Second University of Naples

Street Address

Via L. De Crecchio, 7

Zip code

80138

City

Naples

Province/State

Naples

Country

Italy

Chief of the Host Institution

Prof. Francesco Bresciani

Overhead % 5

Applicant

Position title

Associate Professor

Permanent position:

Yes

Main research fields

Study of the molecular bases of muscular dystrophies

Name of the Laboratory

Patologia Genetica

Chief of the Laboratory

Vincenzo Nigro

Number of staff members 6

Facilities and resources

Clinical resources

Ambulatory of medical genetics

Laboratory space

82mq + common facilities

Computer equipment

3 PC

Office space

2 offices, total 25mq

Major Equipment

HT–DHPLC with fluorescence detector, ABI3100 Capillary sequencer, 7 PCR thermal cyclers, Robot for PCR preparation and pipetting

Core Facilities and Services

2 DHPLC, microarray, LCM, Real–time PCR

Other

Cover Letter

Cover Letter

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Cover Letter

This form has to be filled in only in case of a Revised Application.

Research plan – Project overview

Research plan – Project overview

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Project overview

Abstract

In the majority of cases (60%), the dystrophin gene is broken by huge deletions that span hundreds of thousands of DNA nucleotides, encompassing one or more exons. In the remaining of cases, elusive mutations have been claimed to be responsible for the DMD and BMD phenotypes. In the last few years, a number of these missing mutations have been detected with a laborious use of quantitative analyses or DNA scanning techniques. The identification of these mutations has a pivotal importance in the reliable definition of the carrier status in females and for prenatal diagnosis. It is therefore unbelievable that, although muscular dystrophy associations have a leading role in the promotion of research against genetic diseases by collecting funds and creating novel laboratories, one third of DMD/BMD patients do not obtain any molecular information about their status. Our proposal is to help all requesting Italian centres that recruit DMD/BMD patients, by offering a complete and free of charge high–throughput testing. We will perform molecular diagnosis on DNA samples including: 1) quantitative DNA analyses of all the 79 exons and promoters of the gene to identify deletion/duplications in patients and deletions/duplications in carriers; 2) small mutation screening of all the promoters, the muscle exons and exon–flanking introns.

Multicentre Studies only – Role and contribution of partner(s) in the project

Multicentre Studies only – Coordination and Management

Keywords (maximum five words)

DHPLC, real–time PCR, dystrophin, elusive mutations

Therapeutic perspective

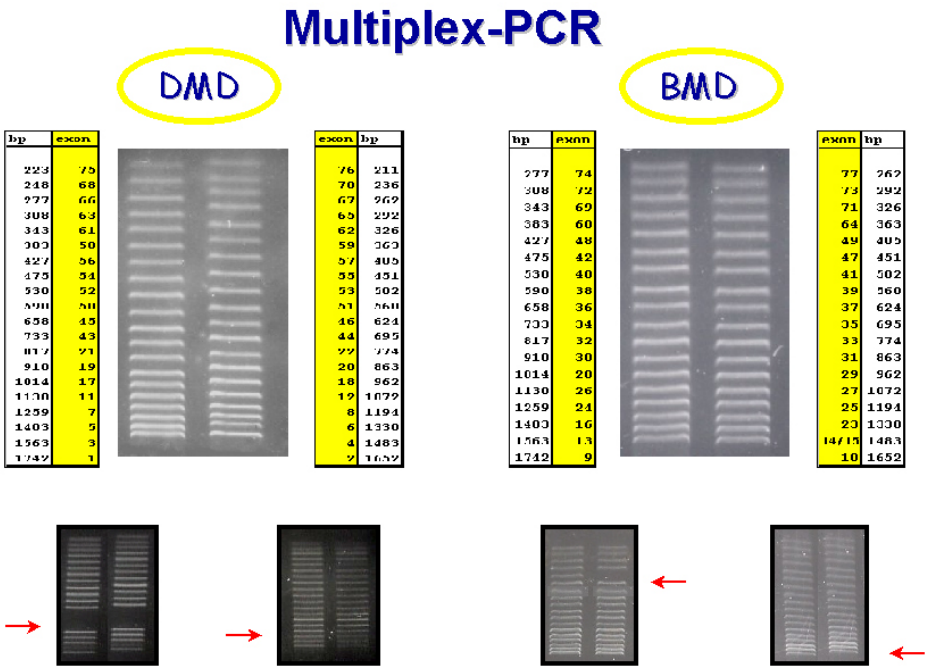
The identification of mutations will be useful for patients who ask to be recruited for gene delivery protocols or for alternative strategies (i.e. aminoglycosides treatment, splicing modifications)

Relevance for Telethon

The majority of the elusive mutations could be easily identifiable by the same people and the same advanced technical devices purchased for research projects, with a very small fund integration. However, most researchers dislike to be involved in diagnosis since diagnostic projects, even if of outstanding technical merits, are not funded i.e. by Telethon. On the other side, diagnostic laboratories choose to perform “easy” tests, such as standard multiplex PCR, for budget requirements.

File attachment

File attached



Research plan – Background

Research plan – Background

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Background

The term muscular dystrophy refers to a group of degenerative muscle diseases characterized by gradual weakening and deterioration of skeletal muscles and frequently the heart and respiratory muscles. One of the major advances in human molecular genetics has been the cloning of the dystrophin gene, encoding a protein absent from muscle in Duchenne muscular dystrophy (DMD) and reduced and/or altered in Becker muscular dystrophy (BMD). DMD result in dramatic physical weakness, so children lose the ability to do things like walk, sit upright, breathe easily, and move their arms and hands. The increasing weakness often leads to other serious complications and a shortened life span. BMD can result in relatively minor physical disabilities or develop later in life. The dystrophin gene maps to the short arm of the X chromosome and it is the longest transcript in the human genome. It spans 2.4 Mb and requires 16 hours to be transcribed. Transcription starts from several different promoters that control the expression of numerous isoforms. The main muscular mRNA, composed of 79 exons, encodes a 427kDa–dystrophin consisting of four distinct domains (N–terminal actin–binding, a larger central rod–like domain, a cysteine–rich domain and the C–terminal domain that binds to the glycoprotein complex).
In the majority of cases (60%), the dystrophin gene is broken by huge deletions that span hundreds of thousands of DNA nucleotides, encompassing one or more exons. In the remaining of cases, atypical deletions, duplications, point mutations or intronic mutations have been claimed to be responsible for the DMD and BMD phenotypes. In the last few years, a number of these missing mutations have been detected with a laborious use of quantitative analyses or DNA scanning techniques . The identification of these mutations has a pivotal importance in the reliable definition of the carrier status in females and for prenatal diagnosis. Unfortunately, although multiplex PCR is a reliable and widespread test to diagnose huge deletions in males, it is not working for the remaining types of mutations. Most families, after many years of study do not know the molecular defect and this is now is becoming a major and urgent requirement.
A complete molecular diagnosis should include: 1) quantitative DNA analysis of all the 79 exons of the gene to identify deletion/duplications in patients and deletions/duplications in carriers; 2) Point mutation screening of all the promoters, the

muscle exons and exon–flanking introns; 3) quantitative and qualitative analysis of dystrophin mRNA from skeletal muscle biopsy.

1) quantitative DNA analysis

Some laboratories propose the quantitative real–time PCR: the method proved to be simple and cost–effective. It is very reliable for direct determination of “known” deletions/duplications in potential DMD/BMD carriers. But quantitative fluorescent PCR of all the exons is not so cheap and the analysis times may become longer. We have set up the complete gene testing in four multiplex PCR reactions (two for DMD and two for BMD) that will be presented in the following sections of this proposal. In our algorithm, real–time is only a second step to confirm the diagnosis using different primer pairs within the deleted/duplicated exons.

2) small mutation screening

Sequencing of PCR products is universally considered as the 'gold standard' for mutation detection. This opinion is due to the fact that sequencing is the only method of mutation identification, but this does not mean it is the best for the first approach to the analysis of a gene. Moreover, even with reduction of sequencing costs (today at Tigem, € 7.50/run), the costs of large screenings by sequencing are unaffordable (i.e., the analysis by double–strand sequencing of 100 PCR fragments in 500 patients will cost € 750,000!). In addition, when a novel putative mutation with uncertain significance is found, this should be checked in a large population of non–affected individuals. These facts immediately indicate that alternative methods to pre–screen and post–screen samples are required. The cheapest screening techniques, like SSCP (single strand conformation polymorphism) and heteroduplex analysis often give unsatisfactory results for their low sensitivities. Therefore, the recurrence of false negatives may invalidate the screening efforts, since mutations can either be truly absent, or go unnoticed in any of the fragments under study. Thus, it could be necessary to re–screen all samples using a different technique. An alternative to these naïve methods is represented by a robust automated technique, DHPLC that we introduced at the Second University since 1999 (thanks to the financial support of the UILDM) and replicated twice at Tigem. In DHPLC, the analysis is performed using a liquid chromatography–based method with a patented reverse–phase column. The test sample is injected onto the column at proper temperature to partially melt the DNA. If there are two DNA species, mutated and normal, the resulting heteroduplexes elute ahead of the homoduplexes. This technique is fully automated and very sensitive (up to 99%). The high–throughput configuration of our instruments requires only a 2–min gradient run for the separation. We added a fluorescence detector. In this configuration DHPLC is able to detect heterozygous mutations in a strong wild–type context. When a subject carries a hemizygous mutation, it is necessary to anneal the sample with a normal control. Instead of using normal controls, we pool DNA from six unrelated patients before PCR, after normalization of the DNA concentrations. This is sufficient to reduce the number and the costs of DHPLC analyses and PCR reactions by at least 84%. Only when a pool were composed of six DNAs with the same mutation, DHPLC analysis would give a false negative result. But this risk is zero, considering the rarity of each single point mutation.

3) analysis of dystrophin mRNA

Patients who had a well–conserved muscle specimen are eligible for dystrophin mRNA studies. This is the most efficient approach, since it detects all mutation types, including those intronic defects that are missed by DNA studies. Furthermore, it is especially suited for the discovery of unknown mutation types in large genes through the usage of novel mechanisms.

Research plan – Specific aims and rationale

Research plan – Specific aims and rationale

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Specific aims and rationale

Rationale
DMD gene was identified eighteen years ago. At that time researchers claimed that this will make immediately possible a direct molecular testing useful for certain carrier identification and prenatal diagnosis. In addition, a future gene replacement therapy was considered realistic. During the course of these years novel technologies have been developed, with important enhancements. After the sequencing of all human genes by the human genome project, sequencing core facilities have greatly benefited from systematic projects and have been able to boost their services by providing longer reads, quicker turnarounds, and reduced costs. It is therefore unbelievable that, although muscular dystrophy associations have a leading role in the promotion of research against genetic diseases by collecting funds and creating novel laboratories, one third of DMD/BMD patients do not obtain any molecular information about their status. These patients cannot be useful for their families. Prevention is still linked to error–prone analyses. Male fetuses run the risk of being erroneously terminated. Alternatively, there is the constant risk of additional cases of disease. This situation is remarkably perverse, since the majority of the mutations could be easily identifiable by the same people and the same advanced technical devices purchased for research projects, with a very small fund integration. However, most researchers dislike to be involved in diagnosis since diagnostic projects, even if of outstanding technical merits, are not funded i.e. by Telethon. On the other side, diagnostic laboratories choose to perform “easy” tests, such as standard multiplex PCR, for budget requirements. Our proposal is to help all requesting Italian centres that recruit DMD/BMD patients, by offering a complete and free of charge high–throughput testing. We will be able to perform the full analysis 300 DNA samples of male patients within two years (120 samples the first year and 180 samples the second year). This will be possible using two different tools: 1) 80plex quantitative PCR 2) the novel generation of DHPLC devices and their forthcoming upgrades.

Specific aims
There is only one aim: to provide maximal technological efforts for robust and reliable molecular diagnosis. We will perform molecular diagnosis on DNA samples including: 1) quantitative DNA analyses of all the 79 exons and promoters of the gene

to identify deletion/duplications in patients and deletions/duplications in carriers; 2) small mutation screening of all the promoters, the muscle exons and exon−flanking introns. In addition, we will ask to send RNA samples, when available to the group of Ferrara, coordinated by dr Alessandra Ferlini, who will perform 3) quantitative and qualitative analysis of dystrophin mRNA from skeletal muscle biopsy.
The proposal is structured according to the following format:
a) DMD/BMD male patients are diagnosed by collaborating centres based on conventional criteria, including symptoms and signs, X−linked inheritance pattern and protein studies. An informed consent is obtained for exclusive use of disease diagnosis. This is a prerequisite for participation.
b) A first−level molecular study is performed by the collaborating laboratories, using multiplex PCR with 30−60 primer pairs, with no result.
c) An adequate amount of DNA or blood samples will be sent to our centre. Each sample will be marked with a secret number code that is converted into an internal lab code.
d) Information about the samples will be delivered using a separate correspondence. This knowledge is required to prevent duplicates of samples. This situation is very common, since most DMD patients, when they do no receive useful results, usually move to other centres in Italy or abroad. For secure management of the privacy, no other laboratory member but the proponent, can identify the name and date of birth of the patients. Files with the codes will never be managed using computers connected to Internet.
e) Quality control of DNA samples will be done, consisting of accurate quantification of genomic DNA, with a fluorometric assay using a reporter dye such as PicoGreen (Molecular Probes), dilution of each individual sample and test of PCR yield.
f) DNA will be first analyzed by the quantitative 80−plex PCR to exclude undetected gross rearrangements, such as undetected atypical deletions or duplications. This technique (see below) is the most efficient one−step tool available, since it detects >80% of all DMD mutations.
g) If such a gross mutation is found, we will communicate the preliminary result. At the same time we will request a separate sample from the same patient to confirm the molecular test using an independent technique and unrelated PCR reactions.
h) If no gross mutation is found, DNA samples will be rechecked for PCR yield and pooled according to our standard procedure for fluorescent DHPLC analysis (see below). Pooled PCR and High−Throughput DHPLC will be performed on all the 79 exons of the dystrophin gene and additional relevant regions (promoters, flanking intronic sequences, etc.).
i) The amplimers showing abnormal profiles will be checked against recurrent polymorphisms by single−base extension technique or by annealing with a polymorphism−bearing amplimer and DHPLC analysis. This situation is very common in several exons, i.e. 45, 48, 59. The polymorphism within the amplimer is easily discriminated from a different change representing a putative mutation.
j) Capillary double−strand sequence analysis will be performed and, only in the case of nontruncating mutations, we will compare the sequence difference with a control population of 500 samples by DHPLC. Control samples will be provided by the DNA bank of the "Servizio di Cardiomiologia e Genetica Medica", Second University of Naples, partner of EuroBioBank Consortium.
k) In the case of a truncating mutation, the procedure will continue as in g)
l) In the case of a putative splice site mutation or in the case of negative results we will request an RNA study together with a western blot assay

m) At each time we will give the opportunity to follow the status of the analysis and to withdraw the sample from the study
n) In the case of females linked to a patient with a known molecular defect, we will also reiterate the test of the male patient accordingly.
o) In the case of females linked to a patient with an unknown molecular defect, we will not perform any test, since a negative result may be improperly evaluated.
p) In the case of females linked to a dead patient, we will perform the test, only if the DNA from an obligate carrier is available.

Research plan – Preliminary results

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Applicant name: Vincenzo Nigro

Preliminary Results

1 – Set up of a novel tool for quantitative study of gross rearrangements
We designed and set up four 20–plex PCR reactions for the quantitative analysis of dystrophin gene rearrangements (see figure). The first two reactions include exons that contain a number of nucleotides that is exact multiple of three and are named (BMD1 and BMD2), since any deletion of these exons cannot alter the reading frame. On the other hand, two more reactions include exons that contain 3n+1 or 3n+2 nucleotides (DMD1 and DMD2). The most relevant features of these multiplex PCR is that we included a calibrated mixture of ten different co–solvents to obtain a linear quantitative amplification. We therefore perform only 19 PCR cycles and intercalate DNA with Gel Star that is at least one order of magnitude more sensitive than ethidium bromide. This allows the accurate determination of DNA amounts to identify duplications. Moreover, the fragment size is dependent on chromosomal order of exons, with 5' exons that produce the largest amplimers, while the 3' end are smaller. The size are spaced according to the logarithm of the molecular weight. With this tool we have identified additional 32 elusive molecular defects (10 deletions and 22 duplications) among a population of 500 DMD/BMD recruited in Naples, at the "Servizio di Cardiomiologia e Genetica Medica", Second University of Naples (Prof. Luisa Politano).

2– Mutation scanning by DHPLC
At present, we manage three High–Throughput DHPLC systems, two at TIGEM and one at the Second University of Naples. This is endowed with an in–line fluorescent detector and micromixer (HSD). We are using the Comparative Mutation Scanning (that selects genes for the presence of aberrant DHPLC profiles not shared by normal controls) for at least 150 selected human genes with the most significant and clear–cut indication to be involved in genetic disorders. At present, we scanned all the exons of the dystrophin gene, including the 3'UTR and 5'UTR and 1,500 bp of genomic sequences upstream of the putative transcription start sites. We set up four PCR reactions for the 5'end, 77+13 for the internal exons (plus flanking introns) and eight for the 3'UTR (total = 102 PCR reactions). For each amplimer, we have defined the predicted denaturation profiles by Wavemaker and Navigator softwares and the

empirical profiles. We amplify each exon using different primers, according to the experimental requirements. The minimal number of primers for each exon is six (three different forward and three different reverse primers). For amplimer usage we take into account the position of very frequent SNPs, to avoid the constant presence of heteroduplexes that could obscure the profiles of novel variations. Primers are all long between 25 and 35 nucleotides to reduce the allele preferences determined by eventual sequence differences located in the region of annealing. This is a very crucial issue for mutation diagnosis. In general each amplimer contains >40 bp of each exon−flanking intronic sequence. Thus, considering an average exon length of 150 bp, a typical PCR reaction yield a fragment of 290 bp (30+40+150+40+30). For this fragment length and considering that the dystrophin gene map to a G−band (AT rich) the sensitivity of HT−DHPLC is very close to 100%. DNA pools were assembled in groups of three, four and up to six different samples. Samples forming a heteroduplex profile are compared with similar number of normal controls. The nature of the changes unique to patients is ascertained by sequencing. PCR are prepared using 384−well microplates with pre−aliquoted DNA samples. The reaction is performed with the use of the buffer we introduced at Tigem mutation detection core. This buffer is required to shorten set−up times, since the amplification is equally efficient under a broad range of cycling temperatures and times. Our attention to cut the costs of each analysis is useful to increase the size of the screening project. In particular, we set up conditions to detect by DHPLC a minority of mutant molecules in a wild type environment. This is possible by a combination of factors that include the use of a proofreading enzyme, a limited number of cycles (less that 25) and the use of an intercalating fluorescent dye. This allow the assembly of large pools of patients as well as of controls. With the present conditions a mutation is always seen by mixing six alleles and is occasionally indistinguishable when you mix more than twelve alleles. With this pooling strategy and assuming to put DNA samples in duplicate, a 384−well plate will be useful to analyze 1152 DNA samples, following the scheme of the table below. For example, when you identify a heterozygous peak in A1 and B2, the mutation is carried by DNA number 2 (the only sample they share). In addition, if a PCR amplification does not work, you can immediately observe the lack of a heterozygous peak in duplicate and you can test the efficiency of the whole process.

A B C D ...
1 1+2+3+4+5+6 7+8+9+10+11+12 13+14+15+16+17+18 19+...+24

2 1+7+13+19+25+31 2+8+14+20+26+32 3+9+...+33 ...

Table − Principle of assembly of 6−plex DNA pools.

In addition to direct mutation scanning, we are planning to look at patients that present homozygosity for all SNPs in certain loci, considering that rarer are the forms, higher is the number of homozygous patients. We are developing these DHPLC−based homozygosity tests that can be applied to all the other autosomal recessive disorders, when no linkage analysis is feasible.
At the present using DHPLC we have identified 41 small mutations in the dystrophin gene.

Research plan – Clinical protocol and methods

Research plan – Clinical protocol and methods

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Clinical protocol and methods

Clinical protocol

Time table

The present proposal is devoted to recognize the primitive molecular defect of DMD/BMD patients, when it remains undetected following the standard analyses. Based on our experience, in Italy the number of new cases is about 90/year, 30 of which remain without molecular diagnosis. After a preliminary evaluation raised with the callaborating centers involved in this project (see collaborations), we estimate that there are at least 450 cases that could be potentially recruited for this study. We propose to study 300 patients (66%) in the first two years of activity (120+180). Based on our analysis of the Naples DNA bank, we expect to find the following defects: 1) a duplication in 15–20% of cases (45–60 cases); 2) an atypical deletion in 4–6% of cases (12–18) ; 3) a small mutation in 40–50% of cases (120–150); 4) a promoter or 3'UTR defect in 2–3% of cases (6–9); 5) no mutation in 25–35% of cases, For these, RNA studies performed by the group of Alessandra Ferlini, will become crucial.

Methods

80–plex
This novel tool allow us to identify up to 80% of DMD/BMD cases in a few hours. The lenghty set up with the use of several different amplimers for the same exon have finally realized the dream to identify all but small changes (see figure) within the DMD gene. Most importantly, the technique is inexpensive and very accurate. Among 400 patients studied no false negative or false positive result was obtained
HT–DHPLC
DHPLC is a well–known technique for mutation discovery studies. DHPLC uses a liquid chromatographic method. This technology, set up by Oefner and Underhill at Stanford University, is based on the discrimination of heteroduplex DNA from homoduplex DNA after an injection onto a patented HPLC column, which uses nonporous poly(styrene–divinylbenzene) copolymers. The small uniform size of the particles (2.1 micron) offers a large surface area. The DNA can bind the column through TEAA (triethyl ammonium acetate) bridging molecules at proper temperature with 0.1 degree of adjustment (usually between 54°C and 68°C). The optimal

temperature is chosen, in order to partially melt the DNA. Using the high−throughput model, the concentration of acetonitrile is increased by 5%/minute at a flow rate of 1.5 ml/min. With this rapid gradient, DNA is gently but quickly extracted from the column. Longer DNA fragments have a higher affinity for the stationary phase due to their higher content of TEAA molecules.
Mutation detection is similar in principle to denaturing gradient gel electrophoresis. Samples who are heterozygous in a single−nucleotide difference have a 1:1 ratio of wild−type and variant DNA. When the PCR fragments are heated and cooled (as in the PCR cycles) mutant DNA helices hybridize with the wild−type ones to form a mixture of homo− and heteroduplexes. The expected ratios are 0.25 for each homoduplex and 0.50 for both heteroduplexes. The high temperature of the column leads to a reduction in the double−stranded portion of the PCR fragment. Single−stranded DNA fragments elute earlier than double−stranded fragments at elevated temperatures. The reason for this is the reduced negative charges in the single−stranded portions of the molecules compared to the double stranded molecule. Therefore, heteroduplex PCR fragments with a higher single stranded content than the homoduplex fragments are retained less than their homoduplex analogues with the heteroduplexes that emerge ahead of the intact homoduplexes.
A column is sequentially loaded with a sample, eluted, and re−equilibrated with buffer for a novel run. Using the High Throughput implementation (HT−3500) the turnaround time can be cut to less than 4 minutes per single fragment analysis, compared with about 9−10 minutes of the past instrument.
The most relevant feature that differentiates DHPLC from all the other mutation scanning methods is that each PCR product can be analyzed directly after a normal amplification, without any treatment or labeling of samples. The main advantages of this method to scan PCR fragments are speed and sensitivity: 96 samples in a standard 8 x 12 format or 384 samples in the new 16x24 format are sequentially loaded, analyzed and all results are stored on a computer hard−disk, without the need for human intervention. The work to be done is to set up conditions prepare solutions and load plates or tubes.
Most importantly, to improve resolution, we use of a DNA polymerase with efficient proofreading activity (Optimase, Transgenomic). This is crucial to analyse larger fragments, since the frequent replication errors by Taq DNA polymerase generate a population of randomly mutated molecules. This gives rise to a round−shaped peak of heteroduplex that can resemble a mutation. The buffer and PCR condition have been optimized to extend the column half−life up to 15,000−18,000 runs.

Research plan – Cited literature

Research plan – Cited literature

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Cited literature

• Online Mendelian Inheritance in Man (OMIM; http://www.ncbi.nlm.nih.gov/Omim) for all mentioned diseases.
• The Leiden Muscular Dystrophy pages© (LDMp; http://www.dmd.nl), a specialized mutation databases for muscular dystrophies.
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• Xiao W, Oefner PJ. Denaturing high−performance liquid chromatography: A review. Hum Mutat. 6: 439−474 (2001)

Personnel

Personnel

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Principal Investigator – Coordinator – Partner

Name

Nigro Vincenzo

Degree

Medicine

Role

P.I. General coordination. Management of confidential data

Birthdate (dd/mm/yyyy) 28/07/1960

Annual effort % 33

Personnel

Name

Aurino Stefania

Degree

Biology

Role

Molecular biologist performing the management of databases

Birthdate (dd/mm/yyyy) 02/09/1976

Annual effort % 50

Is a salary being requested?

No

Personnel

Name

Piluso Giulio

Degree

Biology

Role

molecular biologist performing real–time PCR analyses

Birthdate (dd/mm/yyyy) 13/05/1961

Annual effort % 30

Is a salary being requested?

No

Personnel

Name

Saccone Valentina

Degree

Chemistry and Pharmaceutic Technology

Role

molecular biologist performing DNA quality control and pooling

Birthdate (dd/mm/yyyy) 16/05/1978

Annual effort % 20

Is a salary being requested?

No

Personnel

Name

Esposito Maria

Degree

Technician

Role

molecular biologist performing mutational analysis using DHPLC

Birthdate (dd/mm/yyyy) 16/04/1980

Annual effort % 50

Is a salary being requested?

No

Personnel

Name

Vitiello Carmen

Degree

Medical Biotechnologies

Role

molecular biologist performing polymorphism testing by SBE

Birthdate (dd/mm/yyyy) 28/05/1978

Annual effort % 50

Is a salary being requested?

No

Personnel

Name

to be named

Degree

Chemistry

Role

set up of multiplex PCR reactions

Birthdate (dd/mm/yyyy) 01/01/1975

Annual effort % 100

Is a salary being requested?

Yes

Total Budget

Total Budget

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Requested Budget (Principal Investigator/Coordinator: **Vincenzo Nigro)=** € 150000

Total Budget

This form is the Total Budget report, which automatically summarises all the requested budget specified in the multicenter Applications and which will be available only in the multicenter Applications.

Budget

Budget

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Requested Budget (Principal Investigator/Coordinator: **Vincenzo Nigro)=** € 150000

Budget description

	First year	Second year	Third year	**Total**
Equipment	13000,00	7500,00	0,00	20500,00
Materials, supplies and services	38000,00	52000,00	0,00	90000,00
Salaries and wages	14000,00	15000,00	0,00	29000,00
Travels	1500,00	1500,00	0,00	3000,00
Other	0,00	0,00	0,00	0,00
Overhead	3500,00	4000,00	0,00	7500,00
Total	70000,00	80000,00	0,00	150000,00

Equipment

Description and Justification	**Cost €**
1. 2x384–well thermal cycler	13000,00
2. Dedicated PC	2500,00
3. Photodocumentation	5000,00

Materials, supplies and services

	Description and Justification	Cost €
1.	DHPLC reagents (acetonitrile, columns, TEAA, lamps, etc.)	24000,00
2.	PCR reagents (profreading enzymes, plates, filtertips, etc.)	16000,00
3.	DHPLC servicing (1 instrument for 2 years)	23000,00
4.	Sequencing reactions	12000,00
5.	Real−time and primers	15000,00

Salaries and wages

	Description and Justification	Cost €
1.	one salary for two years for a graduated/technician	29000,00

Travels

	Description and Justification	Cost €
1.	participation to Italian meetings	3000,00

Other

	Description and Justification	Cost €
1.		0,00

Overhead

	Description and Justification	Cost €
1.	Administration by the Department of General Pathology	7500,00

Other Financial Supports

Other Financial Supports

Telethon UILDM Grant number: GUP04008

Project Title: *A high−throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Financial support

Period:

From

To

Gross amount:

Title of the project

Brief description

Granting Agency

Specify overlaps with this application, if any

Collaborations

Collaborations

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Collaborator

Name

Alessandra Ferlini

Department/University/Institution/Laboratory

University of Ferrara – Laboratory of Medical genetics

Actively involved in this Application:

YES

If yes, specify contribution

The group of Alessandra Ferlini will perform RNA studies on muscle biopsy samples. The entire coding sequence is amplified in ten overlapping fragments and a diagnosis shoul be also feasible in the case of deep intronic defects.

Collaborator

Name

Luisa Politano

Department/University/Institution/Laboratory

Dipartimento di Medicina Sperimentale/Seconda Università degli Studi di Napoli/Servizio di Cardiomiologia e Genetica Medica.

Actively involved in this Application:

YES

If yes, specify contribution

The group of Luisa Politano will be involved in genetic counseling and clinical selection of DMD/BMD patients from Southern Italy. It will provide at least 50% of patients.

Collaborator

Name

Enzo Ricci

Department/University/Institution/Laboratory

Department of Neuroscience/Università Cattolica di Roma

Actively involved in this Application:

YES

If yes, specify contribution

The group of Enzo Ricci will be involved in clinical selection of DMD/BMD patients from Center of Italy. It will provide at least 20% of patients.

Collaborator

Name

Carlo Minetti

Department/University/Institution/Laboratory

Department of Paediatrics/Università di Genova/Giannina Gaslini Institute/Neuromuscular Disease Unit

Actively involved in this Application:

YES

If yes, specify contribution

The group of Carlo Minetti will be involved in clinical selection of DMD/BMD patients from North–Western Italy. It will provide at least 5% of patients.

Collaborator

Name

Corrado Angelini

Department/University/Institution/Laboratory

Department of Neurology/Università di Padova

Actively involved in this Application:

YES

If yes, specify contribution

The group of Corrado Angelini will be involved in clinical selection of DMD/BMD patients from North–Eastern Italy. It will provide at least 20% of patients.

Collaborator

Name

Giuseppe Vita

Department/University/Institution/Laboratory

Department of Neurosciences, Psychiatry and Anaestesiology/ Università di Messina

Actively involved in this Application:

YES

If yes, specify contribution

The group of Giuseppe Vita will be involved in clinical selection of DMD/BMD patients from Sicily. It will provide at least 5% of patients.

Suggested reviewers

Suggested reviewers

Telethon UILDM Grant number: GUP04008

Project Title: *A high−throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Reviewer

Name

Lehmann−Horn

Position title

Full professor

Department/University/Institution/Laboratory

Ulm University, Albert−Einstein−Alle 11

City

Ulm

Province/State

Country

Germany

E−mail

frank.lehmann−horn@medizin.uni−ulm.de

Website

Reviewer

Name

Francesco Muntoni

Position title

MD, PhD

Department/University/Institution/Laboratory

Dept. Paediatrics, Hammersmith Hospital, Du Cane Rd

City

London

Province/State

Country

UK

E–mail

f.muntoni@ic.ac.uk

Website

Notes

Notes

Telethon UILDM Grant number: GUP04008

Project Title: *A high−throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Your notes, if any

Lay summary

Lay summary

Telethon UILDM Grant number: GUP04008

Project Title: *A high−throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Lay summary

Project Title (in Italian)

Un servizio ad alta processività per la diagnosi molecolare delle mutazioni "nascoste" del gene della distrofina

Keywords (in Italian)

DHPLC real−time PCR distrofina mutazioni "nascoste"

Project summary for general public (in Italian)

Il gene DMD è stato identificato diciotto anni fa. Allora, i ricercatori ritennero che ciò avrebbe reso immediatamente possibili test diretti di diagnosi molecolare, utili per accertare lo stato di portatrice e per la diagnosi prenatale. Inoltre, si ritenne realisticamente possibile, in futuro, la terapia genica.
Al momento, un terzo dei pazienti DMD/BMD non ottiene alcuna informazione, a livello molecolare, circa il suo stato. Questa situazione è tanto più perversa, giacché la maggioranza delle mutazioni potrebbe essere facilmente identificata dalle stesse persone ed utilizzando le stesse risorse tecnologiche avanzate acquistate per i progetti di ricerca, con una piccolissima integrazione di fondi. Ad ogni modo, la maggior parte dei ricercatori non ama di essere coinvolta nella diagnosi, poiché i progetti diagnostici, anche se di notevole pregio tecnologico, non sono finanziati, ad esempio da Telethon. Dall'altra parte, i laboratori diagnostici scelgono di eseguire test "facili", come la multiplex PCR standard, per esigenze di budget. La nostra proposta è di aiutare tutti i centri italiani richiedenti, che reclutano pazienti DMD/BMD, offrendo un test ad alta processività, completo e gratuito. Noi saremo in grado di eseguire un'analisi completa su 300 campioni di DNA di pazienti maschi entro i primi due anni (120 campioni nel primo anno, e 180 campioni nel secondo). Ciò sarà possibile utilizzando due differenti strumenti: 1) la 80plex PCR quantitativa; 2) la nuova generazione di sistemi DHPLC e la loro futura evoluzione.

Project summary for general public (in English)

DMD gene was identified eighteen years ago. At that time researchers claimed that this will make immediately possible a direct molecular testing useful for certain carrier identification and prenatal diagnosis. In addition, a future gene replacement therapy

was considered realistic. At the present, one third of DMD/BMD patients do not obtain any molecular information about their status. This situation is remarkably perverse, since the majority of the mutations could be easily identifiable by the same people and the same advanced technical devices purchased for research projects, with a very small fund integration. However, most researchers dislike to be involved in diagnosis since diagnostic projects, even if of outstanding technical merits, are not funded i.e. by Telethon. On the other side, diagnostic laboratories choose to perform “easy” tests, such as standard multiplex PCR, for budget requirements. Our proposal is to help all requesting Italian centres that recruit DMD/BMD patients, by offering a complete and free of charge high−throughput testing. We will be able to perform the full analysis 300 DNA samples of male patients within two years (120 samples the first year and 180 samples the second year). This will be possible using two different tools: 1) 80plex quantitative PCR 2) the novel generation of DHPLC devices and their forthcoming upgrades.

Application Summary

Application Summary

Telethon UILDM Grant number: GUP04008

Project Title: *A high–throughput service for the molecular diagnosis of elusive dystrophin gene mutations*

Applicant name: Vincenzo Nigro

Application Summary

Institution: *Department of General Pathology Second University of Naples*

Address: *Via L. De Crecchio, 7*
80138 Naples (Naples)

Type of application: Old Applicant – New Application

Number of sites: *1*

Project duration (years): *2 years*

Principal Investigator/Coordinator: Vincenzo Nigro

Requested Budget (Principal Investigator/Coordinator: **Vincenzo Nigro)=** € 150000

Disease name: *MUSCULAR DYSTROPHY, DUCHENNE TYPE; DMD*

OMIM number: *310200*

Research area: *Neuromuscular disease*