

## A new approach for Next Generation Sequencing in prenatal diagnosis applied to a case of Charcot-Marie-Tooth syndrome.

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In recent years, Next Generation Sequencing (NGS) has become an important tool, not only for gene discovery and research, but also for clinical diagnosis. Genetic diagnosis of rare disorders has developed considerably through the application of massively parallel sequencing methods. To date, a great effort has been made to introduce NGS in noninvasive prenatal testing both for the detection of aneuploidy associated to chromosomes 21, 18 and  $13^{1,2}$  and, in the latest studies, for single gene disorder analysis<sup>3,4</sup>.

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To the best of our knowledge, this is the first report that describes the use of a multi-gene
NGS panel optimized for clinical prenatal diagnosis with a relatively fast turn-around time
that can be multiplexed to allow simultaneous analysis of multiple samples.

In this report the NGPD method was applied for the prenatal genetic diagnosis of Charcot Marie Tooth (CMT) disease in a fetus whose mother was affected by early onset CMT. Of all the types of hereditary sensory-motor polyneuropathy, CMT syndrome is the most frequent. From a clinical point of view, the disorder is characterized by distal muscular atrophy associated with malformation of the feet, osteotendinous hyporeflexia and alterations in sensibility. Based on motor conduction velocity, it is possible to identify type 1 (demyelinating form with a particularly reduced conduction speed) and type 2 (axonal form

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Trio analysis was useful not only for identifying variants, but also for studying inheritance patterns. Variants were sorted for autosomal recessive, autosomal dominant, X-linked and *de novo* mode of inheritance.

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During post-test genetic counseling, the patient was informed both regarding possible penetrance variability of CMT2 and, in case of the syndrome, about the probability of an early onset of pathological signs at same age as the mother (the second year of life). This was a crucial point since the early onset of the syndrome is strictly correlated to its severity. The patient chose to terminate the pregnancy. For further pregnancies, we suggested the patient have preimplantation genetic diagnosis.

The NGPD was very suitable for prenatal diagnosis as it is compatible with the limited quantity and quality of the DNA extracted from the fetal sample through chorionic villous sampling, and, above all, with the times foreseen in prenatal diagnostics; indeed, the results are available after about two weeks and reporting occurred during the fifteenth week of pregnancy. Moreover, the use of a high-throughput NGS platform, NextSeq500, has made a cost reduction possible.

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162	1. Bianchi DW, Parker RL, Wentworth J, et al. DNA sequencing versus standard prenatal
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100	500 011 usonini Obster Gynecol. 2011, 15(5).201 2011. doi:10.1002/u05.15277.

169	3.	Chitty LS, Mason S, Barrett AN, et al. Non-invasive prenatal diagnosis of achondroplasia		
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172	4.	Hill M, Twiss P, Verhoef TI, et al. Non-invasive prenatal diagnosis for cystic fibrosis:		
173		detection of paternal mutations, exploration of patient preferences and cost analysis.		
174		Prenat Diagn. 2015. doi:10.1002/pd.4585.		
175	5.	Filges I, Friedman JM. Exome sequencing for gene discovery in lethal fetal disorders -		
176		harnessing the value of extreme phenotypes. Prenat Diagn. 2014. doi:10.1002/pd.4464.		
177	6.	Talkowski ME, Ordulu Z, Pillalamarri V, et al. Clinical diagnosis by whole-genome		
178		sequencing of a prenatal sample. N Engl J Med. 2012;367(23):2226-2232.		
179		doi:10.1056/NEJMoa1208594.		
180	7.	Casasnovas C, Banchs I, Cassereau J, et al. Phenotypic spectrum of MFN2 mutations in		
181		the Spanish population. J Med Genet. 2010;47(4):249-256.		
182		doi:10.1136/jmg.2009.072488.		
183	8.	Vielhaber S, Debska-Vielhaber G, Peeva V, et al. Mitofusin 2 mutations affect		
184		mitochondrial function by mitochondrial DNA depletion. Acta Neuropathol (Berl).		
185		2013;125(2):245-256. doi:10.1007/s00401-012-1036-y.		
186	9.	Bombelli F, Stojkovic T, Dubourg O, et al. Charcot-Marie-Tooth disease type 2A: from		
187		typical to rare phenotypic and genotypic features. JAMA Neurol. 2014;71(8):1036-1042.		
188		doi:10.1001/jamaneurol.2014.629.		
189				

- 191 **Table 1**
- 192 NGPD panel: genetic diseases are listed on the left and on the right the associated genes
- included in NGPD panel.

GENETIC DISEASE	ASSOCIATED GENES
ACHONDROGENESIS, TYPE IA/TYPE IB	TRIP11/SLC26A2
HYPOCHONDROGENESIS/ACHONDROG	COL2A1
ENESIS, TYPE II	
ACHONDROPLASIA	FGFR3
ALAGILLE SYNDROME 1/TETRALOGY	JAG1
OF FALLOT	
APERT SYNDROME	FGFR2
ATELOSTEOGENESIS, TYPE I	FLNB
ATAXIA-TELANGIECTASIA VARIANT	ATM
BARDET-BIEDL SYNDROME 1	BBS1/BBS10
BLOOM SYNDROME	BLM
CANAVAN DISEASE	ASPA
CARDIOFACIOCUTANEOUS	BRAF
SYNDROME 1	
CHARCOT MARIE TOOTH DISEASE	BSCL2, DNM2, EGR2, FGD4, FIG4, GARS, GDA
	P1, GJB1, HSPB1, HSPB8,KIF1B, LITAF,LMNA,
	MFN2, MPZ, MTMR2, NDRG1, NEFL, PMP22, P
	RPS1, PRX, RAB7A,SBF2, SH3TC2, TRPV4
RHIZOMELIC CHONDRODYSPLASIA	PEX7
PUNCTATA, TYPE 1	

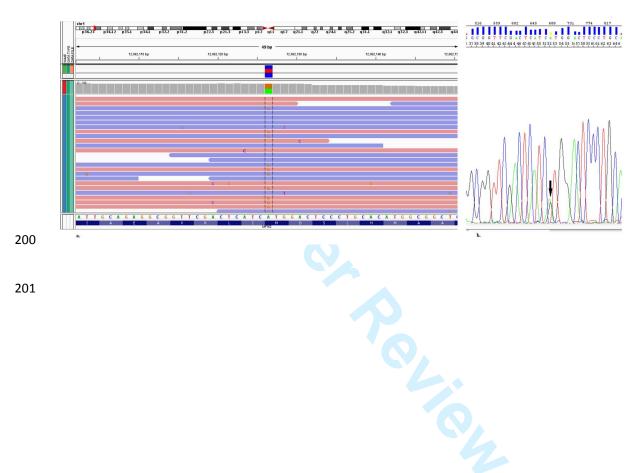
RPS6KA3
NIPBL
SMC1A
HRAS
FGFR2
IKBKAP
TUBB3
SOX9
ADAMTS2/COL1A1/COL1A2/COL3A1/COL5A1/C
OL5A2/PLOD1
EVC
KRT5/KRT14
РАН
CFTR
GALT
TBX5

HYPOCHONDROPLASIA	FGFR3
HYPOPHOSPHATASIA, INFANTILE	ALPL
LISSENCEPHALY, X-LINKED, 1	DCX
LISSENCEPHALY 3	TUBA1A
JOUBERT SYNDROME 3/5/6/8/7/9/2	AHI1/CEP290/TMEM67/ALR13B/RPGRIP1L/CC2
	D2A/TMEM216
KABUKI	KMT2D
MARFAN DISEASE	FBN1
MECKEL SYNDROME	MKS1
MICROCEPHALY	ASPM
MUCOLIPIDOSIS	MCOLN1
NAIL-PATELLA SYNDROME	LMX1B
NOONAN SYNDROME	PTPN11/SOS1/KRAS/RAF1/BRAF/NRAS/CBL
HOLOPROSENCEPHALY	SHH/SIX3
OSTEOGENESIS IMPERFECTA, TIPO	COL1A1/COL1A2/CRTAP/LEPRE1
I/II/III/IV/VII	
POLYMICROGYRIA	TUBB2B
POLYCISTIC KIDNEY DISEASE	PKD1/PKD2/PKHD1
PFEIFFER SYNDROME	FGFR1

RETT SYNDROME	MECP2
SAETHRE-CHOTZEN SYNDROME	TWIST1
SECKEL SYNDROME	ATR
SMITH-LEMLI-OPITZ SYNDROME	DHCR7
DEAFNESS	GJB2/GJB6
SOTOS SYNDROME	NSD1
TAY-SACHS DISEASE	HEXA
TIROSINEMIA	FAH
TREACHER COLLINS SYNDROME	TCOF1
WILSON SYNDROME	ATP7B
ZELLWEGER SYNDROME	PEXI
<u></u>	

# 196 Figure 1

- 197 *MFN2* c.1126A>G on CVS sample: a. NGS result displayed by Integrative Genome Viewer
- 198 (IGV); b. variant confirmation by Sanger sequencing.



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- 5. Filges I, Friedman JM. Exome sequencing for gene discovery in lethal fetal disorders -176 harnessing the value of extreme phenotypes. Prenat Diagn. 2014. doi:10.1002/pd.4464. 177
- 178 6. Talkowski ME, Ordulu Z, Pillalamarri V, et al. Clinical diagnosis by whole-genome 179 sequencing of a prenatal sample. N Engl J Med. 2012;367(23):2226-2232. 180 doi:10.1056/NEJMoa1208594.
- 7. Casasnovas C, Banchs I, Cassereau J, et al. Phenotypic spectrum of MFN2 mutations in 181 the Spanish population. Med 2010;47(4):249-256. 182 JGenet. doi:10.1136/jmg.2009.072488. 183
- 8. Vielhaber S, Debska-Vielhaber G, Peeva V, et al. Mitofusin 2 mutations affect 184 mitochondrial function by mitochondrial DNA depletion. Acta Neuropathol (Berl). 185 2013;125(2):245-256. doi:10.1007/s00401-012-1036-y. 186
- Bombelli F, Stojkovic T, Dubourg O, et al. Charcot-Marie-Tooth disease type 2A: from 187 9. typical to rare phenotypic and genotypic features. JAMA Neurol. 2014;71(8):1036-1042. 188 doi:10.1001/jamaneurol.2014.629. 189

190

## 192 **Table 1**

- 193 NGPD panel: genetic diseases are listed on the left and on the right the associated genes
- included in NGPD panel.

GENETIC DISEASE	ASSOCIATED GENES	]
ACHONDROGENESIS, TYPE IA/TYPE IB	TRIP11/SLC26A2	Formatted: Font: Italic
,	· · · · · · · · · · · · · · · · · · ·	Formatted: Font: Not Bold, Italic
HYPOCHONDROGENESIS/ACHONDROG	COL2A1	Formatted: Font: Italic
ENESIS, TYPE II		Formatted: Font: Not Bold, Italic
ACHONDROPLASIA	FGFR3	Formatted: Font: Italic
	· · · · · · · · · · · · · · · · · · ·	Formatted: Font: Not Bold, Italic
ALAGILLE SYNDROME 1/TETRALOGY	JAGI	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
OF FALLOT		
APERT SYNDROME	FGFR2	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
ATELOSTEOGENESIS, TYPE I	FLNB	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
ATAXIA-TELANGIECTASIA VARIANT	ATM	Formatted: Font: Italic
DADDET DIEDI GYNDDOME 1		Formatted: Font: Not Bold, Italic
BARDET-BIEDL SYNDROME 1	BBS1/BBS10	Formatted: Font: Italic
BLOOM SYNDROME	BLM	Formatted: Font: Not Bold, Italic
		Formatted: Font: Italic
CANAVAN DISEASE	ASPA	Formatted: Font: Not Bold, Italic
		Formatted: Font: Italic
CARDIOFACIOCUTANEOUS	BRAF	Formatted: Font: Not Bold, Italic
		Formatted: Font: Italic
SYNDROME 1		Formatted: Font: Not Bold, Italic
CHARCOT MARIE TOOTH DISEASE	BSCL2, DNM2, EGR2, FGD4, FIG4, GARS, GDA-	Formatted: Font: Italic
	P1, GJB1, HSPB1, HSPB8,KIF1B, LITAF,LMNA,	
	MFN2, MPZ, MTMR2, NDRG1, NEFL, PMP22, P	
	RPS1, PRX, RAB7A,SBF2, SH3TC2, TRPV4	Formatted: Font: Not Bold, Italic
RHIZOMELIC CHONDRODYSPLASIA	PEX7	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
RHIZOMELIC CHONDRODYSPLASIA PUNCTATA, TYPE 1		Formatted: Font: Italic

COFFIN-LOWRY SYNDROME	RPS6KA3	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
CORNELIA DE LANGE SYNDROME 1	NIPBL	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
CORNELIA DE LANGE SYNDROME 2	<u>SMC1A</u>	Formatted: Font: Italic
COSTELLO SYNDROME	HRAS	Formatted: Font: Not Bold, Italic
COSTELLO STINDROME		Formatted: Font: Italic
CROUZON SYNDROME	FGFR2	Formatted: Font: Not Bold, Italic
		Formatted: Font: Italic
DYSAUTONOMIA, FAMILIAL	IKBKAP.	Formatted: Font: Not Bold, Italic
		Formatted: Font: Italic
CORTICAL DYSPLASIA, COMPLEX	TUBB3	Formatted: Font: Not Bold, Italic
		Formatted: Font: Italic
WITH OTHER BRAIN		Formatted: Font: Not Bold, Italic
MALFORMATIONS 1		
CAMPOMELIC DYSPLASIA WITH	SOX9	Coursetted, Copt. Italia
CAMPOMELIC DISPLASIA WIT		Formatted: Font: Italic
AUTOSOMAL SEX REVERSAL		Formatted: Font: Not Bold, Italic
No robolini e sex ke veksi te		
EHLERS-DANLOS SYNDROME, TYPE	ADAMTS2/COLIA1/COLIA2/COL3A1/COL5A1/C-	Formatted: Font: Italic
,	·	
VIIC/TYPE I/AUTOSOMAL RECESSIVE	OL5A2/PLODI	Formatted: Font: Not Bold, Italic
CARDIAC VALVULAR FORM/TYPE		
IV/TYPEVI		
ELLIS-VAN CREVELD SYNDROME	EVC	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
EPIDERMOLYSIS BULLOSA SIMPLEX	KRT5/KRT14	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
DOWLING-MEARA		
TYPE/GENERALIZED		
	DAIL	Formatted: Font: Italic
PHENYLKETONURIA	РАН	
		Formatted: Font: Not Bold, Italic
PHENYLKETONURIA CYSTIC FIBROSIS	CFTR	Formatted: Font: Not Bold, Italic Formatted: Font: Italic
CYSTIC FIBROSIS	<u>CFTR</u>	Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic
		Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic
CYSTIC FIBROSIS	<u>CFTR</u>	Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic
CYSTIC FIBROSIS GALACTOSEMIA	CFTR GALT	Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic
CYSTIC FIBROSIS GALACTOSEMIA	CFTR GALT	Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic
CYSTIC FIBROSIS GALACTOSEMIA HOLT-ORAM SYNDROME HYPOCHONDROPLASIA	CFTR GALT TBX5 FGFR3	Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic
CYSTIC FIBROSIS GALACTOSEMIA HOLT-ORAM SYNDROME	CFTR GALT TBX5	Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Italic
CYSTIC FIBROSIS GALACTOSEMIA HOLT-ORAM SYNDROME HYPOCHONDROPLASIA	CFTR GALT TBX5 FGFR3	Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic Formatted: Font: Not Bold, Italic Formatted: Font: Italic

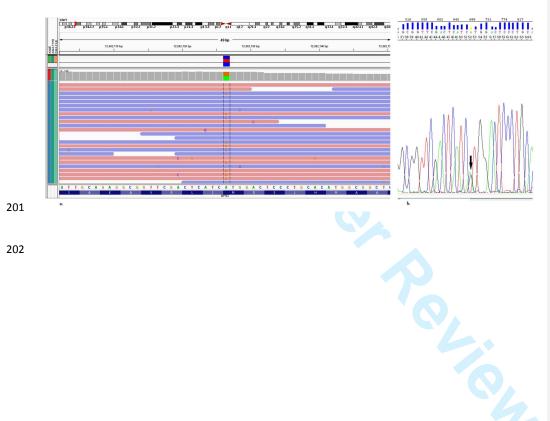
LISSENCEPHALY, X-LINKED, 1	DCX	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
LISSENCEPHALY 3	TUBAIA	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
JOUBERT SYNDROME 3/5/6/8/7/9/2	AHII/CEP290/TMEM67/ALR13B/RPGRIP1L/CC2	Formatted: Font: Italic
	D2A/TMEM216	Formatted: Font: Not Bold, Italic
KABUKI	KMT2D	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
MARFAN DISEASE	FBN1	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
MECKEL SYNDROME	MKSI	Formatted: Font: Italic
		Formatted: Font: Not Bold, Italic
MICROCEPHALY	ASPM	Formatted: Font: Italic
	MCOLNI	Formatted: Font: Not Bold, Italic
MUCOLIPIDOSIS	MCOLNI	Formatted: Font: Italic
NAIL-PATELLA SYNDROME	LMXIB	Formatted: Font: Italic
NOONAN SYNDROME	PTPN11/SOS1/KRAS/RAF1/BRAF/NRAS/CBL	Formatted: Font: Italic
HOLOPROSENCEPHALY	SHH/SIX3	Formatted: Font: Italic
OSTEOGENESIS IMPERFECTA, TIPO	COLIAI/COLIA2/CRTAP/LEPRE1	Formatted: Font: Italic
I/II/III/IV/VII		
POLYMICROGYRIA	TUBB2B	Formatted: Font: Italic
POLYCISTIC KIDNEY DISEASE	PKD1/PKD2/PKHD1	Formatted: Font: Italic
PFEIFFER SYNDROME	FGFR1	Formatted: Font: Italic
RETT SYNDROME	MECP2	Formatted: Font: Italic
SAETHRE-CHOTZEN SYNDROME	_TWIST1	Formatted: Font: Italic
SECKEL SYNDROME	ATR	Formatted: Font: Italic
SMITH-LEMLI-OPITZ SYNDROME	DHCR7	Formatted: Font: Italic

DEAFNESS	GJB2/GJB6	 Formatted: Font: Italic
SOTOS SYNDROME	NSD1	 Formatted: Font: Italic
TAY-SACHS DISEASE	HEXA	
TAT-SACHS DISEASE		 Formatted: Font: Italic
TIROSINEMIA	FAH	 Formatted: Font: Italic
TREACHER COLLINS SYNDROME	TCOF1	 Formatted: Font: Italic
WILSON SYNDROME	ATP7B	 Formatted: Font: Italic
ZELLWEGER SYNDROME	PEXI	 Formatted: Font: Italic
	0	-

### 197 Figure 1



199 (IGV); b. variant confirmation by Sanger sequencing.





MFN2 c.1126A>G: a. NGS result displayed by Integrative Genome Viewer (IGV); b. variant confirmation by Sanger sequencing.

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