Published in final edited form as:

Hum Mutat. 2010 May ; 31(5): E1319–E1331. doi:10.1002/humu.21239.

Novel *TMEM67* Mutations and Genotype-phenotype Correlates in Meckelin-related Ciliopathies

Miriam Iannicelli[1,#], **Francesco Brancati**[1,2,#], **Soumaya Mougou-Zerelli**[3], **Annalisa Mazzotta**[1], **Sophie Thomas**[3], **Nadia Elkhartoufi**[4], **Lorena Travaglini**[1], **Céline Gomes**[3], **Gian Luigi Ardissino**[5], **Enrico Bertini**[6], **Eugen Boltshauser**[7], **Pierangela Castorina**[5], **Stefano D'Arrigo**[8], **Rita Fischetto**[9], **Brigitte Leroy**[10], **Philippe Loget**[11], **Maryse Bonnière**[12], **Lena Starck**[13], **Julia Tantau**[14], **Barbara Gentilin**[5], **Silvia Majore**[15], **Dominika Swistun**[16], **Elizabeth Flori**[17], **Faustina Lalatta**[5], **Chiara Pantaleoni**[8], **Johannes.Penzien**[18], **Paola Grammatico**[15], **the International JSRD Study Group**, **Bruno Dallapiccola**[6], **Joseph G. Gleeson**[16], **Tania Attie-Bitach**[3,4,*], and **Enza Maria Valente**[1,19,*]

[*]Correspondence to: Enza Maria Valente, Neurogenetics Unit, CSS-Mendel Institute, viale Regina Margherita 261 I-00198 Rome, Italy; Tel.: +39 06 4416 0537; Fax: +39 06 4416 0548; e.valente@css-mendel.it; or to: Tania Attie-Bitach, tania.attie@inserm.fr.
[#]*These authors contributed equally to this work.*

INTERNATIONAL JSRD STUDY GROUP

Other members of the International JSRD Study Group are: L. Ali Pacha, M. Tazir (Algiers, Algeria); A. Zankl (Herston, Australia); R. Leventer (Parkville, Australia); P. Grattan-Smith (Sydney, Australia); A. Janecke (Innsbruck, Austria); M. D'Hooghe (Brugge, Belgium); Y. Sznajer (Bruxelles, Belgium); R. Van Coster (Ghent, Belgium); L. Demerleir (Brussels, Belgium); K. Dias, C. Moco, A. Moreira (Porto Alegre, Brazil); C. Ae Kim (Sao Paulo, Brazil); G. Maegawa (Toronto, Canada); D. Loncarevic, V. Mejaski-Bosnjak, D. Petkovic (Zagreb, Croatia); G.M.H. Abdel-Salam, A. Abdel-Aleem, M.S. Zaki (Cairo, Egypt); I. Marti, S. Quijano-Roy (Garches, France); S. Sigaudy (Marseille, France); P. de Lonlay, S. Romano, A. Verloes (Paris, France); R. Touraine (St. Etienne, France); M. Koenig, C. Lagier-Tourenne, J. Messer (Strasbourg, France); P. Collignon (Toulon, France); N. Wolf (Heidelberg, Germany); H. Philippi (Mainz, Germany); J. Lemke (Tubingen, Germany); C. Dacou-Voutetakis, S. Kitsiou Tzeli, R. Pons (Athens, Greece); L. Sztriha (Szeged, Hungary); S. Halldorsson, J. Johannsdottir, P. Ludvigsson (Reykjavik, Iceland); S. R. Phadke (Lucknow, India); V. Udani (Mumbay, India); B. Stuart (Dublin, Ireland); A. Magee (Belfast, Northern Ireland); D. Lev, M. Michelson (Holon, Israel); B. Ben-Zeev (Ramat-Gan, Israel); M. Di Giacomo, M. Gentile, G. Guanti, O. D'Addato, F. Papadia, M. Spano (Bari, Italy); F. Bernardi, M. Seri (Bologna, Italy); F. Benedicenti, F. Stanzial (Bolzano, Italy); R. Borgatti (Bosisio Parini, Italy); P. Accorsi, S. Battaglia, E. Fazzi, L. Giordano, C. Izzi, L. Pinelli (Brescia, Italy); L. Boccone (Cagliari, Italy); P. Guanciali (Chieti, Italy); R. Romoli (Como, Italy); S. Bigoni, A. Ferlini (Ferrara, Italy); E. Andreucci, M.A. Donati, M. Genuardi (Florence, Italy); G. Caridi, M.T. Divizia, F. Faravelli, G. Ghiggeri, A. Pessagno (Genoa, Italy); M. Amorini, M. Briguglio, S. Briuglia, L. Rigoli, C. Salpietro, G. Tortorella (Messina, Italy); A. Adami, G. Marra, D. Riva, B. Scelsa, L. Spaccini, G. Uziel (Milan, Italy); G. Coppola, E. Del Giudice, G. Vitiello (Naples, Italy); A.M. Laverda, K. Ludwig, A. Permunian, A. Suppiej (Padova, Italy); C. Macaluso (Parma, Italy); S. Signorini, C. Uggetti (Pavia, Italy); R. Battini (Pisa, Italy); M. Di Giacomo (Potenza, Italy); M. Priolo (Reggio Calabria, Italy); M.R. Cilio, A. D'Amico, M.L. Di Sabato, F. Emma, V. Leuzzi, P. Parisi, G. Stringini, G. Zanni (Rome, Italy); M. Pollazzon, A. Renieri, M. Vascotto (Siena, Italy); M. Silengo (Torino, Italy); R. De Vescovi (Trieste, Italy); D. Greco, C. Romano (Troina, Italy); M. Cazzagon (Udine, Italy); A. Simonati (Verona, Italy); A.A. Al-Tawari, L. Bastaki, (Kuwait City, Kuwait); A. Mégarbané (Beirut, Lebanon); A. Matuleviciene (Vilnius, Lithuania); V. Sabolic Avramovska (Skopje, Macedonia); E. Said (Msida, Malta); M.M. de Jong (Groningen, The Netherlands); T. Prescott, P. Stromme, C. von der Lippe (Oslo, Norway); R. Koul, A. Rajab (Muscat, Oman); M. Azam (Islamabad, Pakistan); C. Barbot (Oporto, Portugal); B. Jocic-Jakubi (Nis, Serbia); B. Gener Querol (Baracaldo, Spain); L. Martorell Sampol (Barcelona, Spain); B. Rodriguez (La Coruna, Spain); I. Pascual-Castroviejo (Madrid, Spain); S. Strozzi (Bern, Switzerland); J. Fluss (Geneva, Switzerland); S. Teber, M. Topcu (Ankara, Turkey); B. Anlar, S. Comu, E. Karaca, H. Kayserili, A. Yüksel (Istanbul, Turkey); M. Akgul (Izmir, Turkey); M. Akcakus (Kayseri, Turkey); L. Al Gazali (Al Ain, UAE); D. Nicholl (Birmingham, UK); C.G. Woods (Cambridge, UK); C. Bennett, J. Hurst, E. Sheridan (Leeds, UK); A. Barnicoat, L. Carr, R. Hennekam, M. Lees, F. McKay (London, UK); L. Yates (Newcastle-upon-Tyne, UK); E. Blair (Oxford, UK); S. Bernes (Mesa, Arizona, US); H. Sanchez (Fremont, California, US); A.E. Clark (Laguna Niguel, California, US); E. DeMarco, C. Donahue, E. Sherr (San Francisco, California, US); J. Hahn, T.D. Sanger (Stanford California, US); T.E. Gallager (Manoa, Hawaii, US); W.B. Dobyns (Chicago, Illinois, US); C. Daugherty (Bangor, Maine, US); K.S. Krishnamoorthy, D. Sarco, C.A. Walsh (Boston, Massachusetts, US); T. McKanna (Grand Rapids, Michigan, US); J. Milisa (Albuquerque, New Mexico, US); W.K. Chung, D.C. De Vivo, H. Raynes, R. Schubert (New York, New York, US); A. Seward (Columbus, Ohio, US); D.G. Brooks (Philadephia, Pennsylvania, US); A. Goldstein (Pittsburg, Pennsylvania, US); J. Caldwell, E. Finsecke (Tulsa, Oklahoma, US); B.L. Maria (Charleston, South Carolina, US), K. Holden (Mt. Pleasant, South Carolina, US); R.P. Cruse (Houston, Texas, US); K.J. Swoboda, D. Viskochil (Salt Lake City, Utah, US).

[1]Casa Sollievo della Sofferenza Hospital, San Giovanni Rotondo and CSS-Mendel Institute, Rome, Italy [2]CeSI, Aging Research Centre, and Dept. of Biomedical Sciences, G. d'Annunzio University Foundation, Chieti, Italy [3]INSERM U-781, Hôpital Necker-Enfants Malades and Université René Descartes, Paris V, France [4]Dept of Genetics, Hôpital Necker-Enfants Malades, Assistance Publique Hôpitaux de Paris (APHP), Paris, France [5]Foundation IRCCS Ospedale Maggiore Policlinico Mangiagalli e Regina Elena, Milan, Italy [6] IRCCS Ospedale Pediatrico Bambino Gesù, Rome, Italy [7]Dept. of Neurology, Children's University Hospital, Zurich, Switzerland [8]Division of Neurologia dello Sviluppo, Carlo Besta Neurologic Institute Foundation, Milan, Italy [9]UO Metabolic Disease-Medical Genetics, PO Giovanni XXIII-AOU Policlinico Consorziale, Bari, Italy [10]Service d'Anatomie et Cytologie Pathologiques, CHI de Poissy, Saint Germain-en-Laye [11]Cabinet d'Anatomie et Cytologie Pathologiques Richier, Rennes, France [12]Anatomopathologie, Lille, France [13]Sachs'Children's Hospital, Stockholm,Sweden [14]Service d'Anatomopathologie, Hôpital Saint-Vincent de Paul, AP-HP, Paris [15] Dept. of Experimental Medicine, Sapienza University,S.Camillo-Forlanini Hospital, Rome, Italy [16]Neurogenetics Laboratory, Howard Hughes Medical Institute, Dept. of Neurosciences, University of California, San Diego, La Jolla (CA), USA [17]Service de cytogénétique, Fédération de génétique, Hôpitaux Universitaires de Strasbourg, Strasbourg, France [18]Children's Hospital, Augsburg, Germany [19]Dept. of Medical and Surgical Pediatric Sciences, University of Messina, Messina, Italy.

Abstract

Human ciliopathies are hereditary conditions caused by defects of proteins expressed at the primary cilium. Among ciliopathies, Joubert syndrome and related disorders (JSRD), Meckel syndrome (MKS) and nephronophthisis (NPH) present clinical and genetic overlap, being allelic at several loci. One of the most interesting gene is *TMEM67*, encoding the transmembrane protein meckelin. We performed mutation analysis of *TMEM67* in 341 probands, including 265 JSRD representative of all clinical subgroups and 76 MKS fetuses. We identified 33 distinct mutations, of which 20 were novel, in 8/10 (80%) JS with liver involvement (COACH phenotype) and 12/76 (16%) MKS fetuses. No mutations were found in other JSRD subtypes, confirming the strong association between *TMEM67* mutations and liver involvement. Literature review of all published *TMEM67* mutated cases was performed to delineate genotype-phenotype correlates. In particular, comparison of the types of mutations and their distribution along the gene in lethal versus non lethal phenotypes showed in MKS patients a significant enrichment of missense mutations falling in *TMEM67* exons 8 to 15, especially when in combination with a truncating mutation. These exons encode for a region of unknown function in the extracellular domain of meckelin.

Keywords

TMEM67; *MKS3*; Joubert syndrome; Meckel syndrome; congenital hepatic fibrosis; COACH syndrome

INTRODUCTION

Human ciliopathies represent an expanding group of autosomal or X-linked disorders caused by defects of proteins expressed at the primary cilium or its apparatus (Hildebrandt and Zhou, 2007; Sharma et al., 2008). Primary cilia are microtubule-based structures found in most tissues, that function as cellular sensors and control axonal migration and cell polarity during development (Gerdes et al., 2009; Marshall, 2008). The wide expression pattern of ciliary proteins well explains the multiorgan involvement seen in most ciliary disorders, mainly implicating the central nervous system (CNS), retina, kidneys and liver (Lancaster and Gleeson, 2009).

There is striking clinical and genetic overlap among some ciliopathies, namely Joubert syndrome and related disorders (JSRD; MIM# 213300), Meckel syndrome (MKS; MIM# 249000), and Nephronophthisis (NPH; MIM# 256100). These conditions are known to be allelic at several gene loci, albeit genotype-phenotype correlations are only partly understood (Lancaster and Gleeson, 2009). Among these genes, great interest has recently arisen on *TMEM67* (MIM# 609884) that encodes meckelin (Dawe et al., 2007; Smith et al., 2006). This is a 995 aminoacid protein, with an extracellular N-terminus containing a signal peptide and a cysteine rich domain, a transmembrane portion and an intracellular C-terminus including a coiled-coil domain (Khaddour et al., 2007; Smith et al., 2006).

TMEM67 was first identified as causative of MKS (Smith et al., 2006), a lethal disorder displaying CNS malformations (typically occipital encephalocele), multicystic kidneys, ductal plate dysplasia with congenital hepatic fibrosis (CHF) and postaxial polydactyly (Salonen, 1984; Salonen and Paavola, 1998). Large mutation screenings identified *TMEM67* mutations in 23 of 195 (12%) MKS fetuses (Baala et al., 2007; Consugar et al., 2007; Khaddour et al., 2007; Smith et al., 2006; Tallila et al., 2009), including some fetuses with Meckel-like phenotypes. These lacked at least one MKS diagnostic criterion, and their brain pathology often resembled the “molar tooth sign” (MTS) (Baala et al., 2007). The MTS defines a specific constellation of cerebellar and brainstem abnormalities that is peculiar of JSRD, another heterogeneous group of ciliopathies with CNS, retinal, renal and hepatic manifestations (Valente et al., 2008). Within the JSRD spectrum, there is strong correlation between *TMEM67* mutations and the subgroup of JS plus liver involvement (COACH syndrome; MIM# 216360), with an overall mutation frequency of 73% (Baala et al., 2007; Brancati et al., 2009; Doherty et al., 2009).

Two additional *TMEM67*-related ciliopathies were recently delineated. Otto and collaborators reported mutations in five of 62 (8%) probands with NPH and CHF (Otto et al., 2009). Finally, *TMEM67* was found mutated in two families with a peculiar association of polycystic kidney (mimicking autosomal recessive polycystic kidney disease - ARPKD), NPH, CHF and midbrain-hindbrain abnormalities within the MTS spectrum, defined as ARPKD-like syndrome (Gunay-Aygun et al., 2009).

This extreme clinical heterogeneity associated with mutations in one and the same gene is intriguing, and calls for the delineation of specific clinical-genetic correlates. The allelic spectrum of *TMEM67* is broad and includes missense, truncating and splice site mutations, as well as rare multiexon deletions. A preliminary correlation has been observed between truncating mutations and the occurrence of MKS, while missense mutations are more frequently detected in association to less severe phenotypes such as COACH and NPH. However, a systematic analysis of the phenotypic burden of *TMEM67* mutations has not been performed yet.

Here, we present the molecular screening of *TMEM67* in two cohorts of patients, including JSRD cases representative of all clinical subgroups, and MKS fetuses. We describe and characterize 20 novel mutations, perform a detailed review of all published mutations and discuss genotype–phenotype correlates.

MATERIALS AND METHODS

Patients

A total of 341 probands were analyzed. The first group included 265 probands with clinically and neuroradiologically confirmed diagnosis of JSRD. Detailed clinical data were obtained by referring clinicians of the International JSRD Study Group through a standardized questionnaire, allowing to assign patients to the following subgroups: pure JS

(n=101), JS plus retinopathy (n=51), JS plus renal disease (n=11), cerebello-oculo-renal syndrome (n=80), JS plus liver disease (n=10), oro-facio-digital syndrome type VI (n=12). The second group included 76 fetuses diagnosed with MKS according on established criteria (Salonen, 1984). Pregnancies were terminated after genetic counseling, in accordance with local laws. Subjects were recruited worldwide and collected under approved institutional human subject protocols. Written informed consent was obtained from all families. A number of these patients have been included in previous mutational screenings of other JSRD/MKS causative genes, while probands already tested for *TMEM67* and previously described (Brancati et al., 2009; Khaddour et al., 2007) have been excluded from the study.

Mutational analysis

Exons and exon-intron junctions of the *TMEM67* gene were searched for mutations adopting two distinct strategies. In the cohort of JSRD patients, the High Resolution Melting technique (HRM) on a LightCycler® 480 Real-Time PCR system (Roche Applied Science) was used to analyze DNA samples of both parents of each affected proband, in order to overcome the limitations of HRM technique in discriminating homozygous mutations (Wittwer, 2009). Each PCR reaction included 15-25 ng of genomic DNA and High Resolution Master Mix (Roche Applied Science), which contained FastStart Taq DNA polymerase, reaction buffer, dNTPs mix and ResoLight Dye. $MgCl_2$ and each primer were added to 3mM and 0.2μM final concentrations respectively. Primers have been reported previously (Brancati et al., 2009). PCR conditions included an initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation (95°C for 15 s), annealing (primer dependent for 15 s) and extension (72°C for 15 s), with a final extension at 72°C for 2 m. After PCR, a post-amplification melting curve program was initiated by heating to 95°C and cooling to 40°C for 1 min each, and then increasing the temperature to 95°C while continuously measuring fluorescence at 25 acquisitions per degree. Melting curves were analyzed by LightCycler 480 Gene Scanning software (Roche Applied Science). Samples with significant difference in melting profiles and corresponding proband samples underwent direct bidirectional sequencing using the Big Dye Terminator Chemistry and an ABI 3100 Capillary Array Sequencer (Applied Biosystems). In the cohort of MKS patients, bidirectional sequencing was performed as previously described (Khaddour et al., 2007).

Bioinformatic analysis

DNA mutation numbering was based on cDNA sequence, +1 being the first nucleotide of the ATG translation initiation codon in the reference sequence. Mutation description was checked with Mutalyzer software (http://www.humgen.nl/mutalyzer/1.0.1). Prediction of the possible impact of missense variants on the function of meckelin protein was obtained with PolyPhen software (http://genetics.bwh.harvard.edu/pph/), while HSF (http://www.umd.be/HSF) was used to evaluate the potential impact of nucleotide changes on splicing. Multiple sequence alignments of the human meckelin protein and its orthologues were generated using the ClustalW program (http://www.ebi.ac.uk/clustalw/). Accession numbers are as follows: human *TMEM67* mRNA sequence: NM_153704.5; meckelin protein sequence: Homo sapiens NP_714915.3 or ENSP00000314488; Macaca mulatta ENSMMUP00000007350; Rattus norvegicus ENSRNOP00000021839; Mus musculus ENSMUSP00000052644; Gallus gallus ENSGALP00000025642; Tetraodon nigris GSTENP00034026001; Drosophila melanogaster FBpp0112166; Caenorhabditis elegans F35D2.4. Mutation frequencies were compared using a two-tail Fisher's exact test, with correction for multiple tests when applicable. Significance was set at $p<0.05$.

RESULTS

The molecular analysis identified 33 distinct mutations (20 novel and 13 previously reported) in 20 out of 341 families. Overall, 12 mutations were truncating (including frameshift and nonsense), three affected 5' or 3' canonical splice-sites and 18 were missense. All mutations segregated with the disease in familial cases. None of the nine newly identified missense mutations were found in 200 control chromosomes, and alignment with meckelin orthologues showed all affected residues to be conserved among different species (Figure 1). Detailed clinical features of mutated JSRD patients and MKS fetuses are summarized in Tables 1 and 2.

Pathogenic mutations in JSRD

Among the 265 JSRD patients, *TMEM67* compound heterozygous mutations were identified in 8 out of 10 (80%) probands with a phenotype of JS plus liver disease. Twelve mutations were missense, three truncating and one affected splicing. Liver involvement varied from a clinically mild picture of asymptomatic but constant elevation of liver enzymes to more severe presentations leading to liver insufficiency and biopsy-proven CHF. Six of the eight probands had mono-or bilateral colobomas, while the affected sister of one proband with bilateral colobomas had optic nerve hypoplasia. Only one patient (COR212) presented post-axial polydactyly of the four limbs and infantile NPH, that progressed to chronic renal failure at age six months. Several patients were too young at latest examination to rule out the possibility that some of them would develop juvenile NPH later in life. Biallelic mutations were not detected in any of the 255 patients representative of the other JSRD phenotypes.

Pathogenic mutations in MKS

In this group, we identified 18 distinct mutations in 12 out of 76 (16%) MKS fetuses, either in homozygosity (n=5) or compound heterozygosity (n=7). Of these mutations, seven were missense (one recurrent in two fetuses), nine truncating and two affected splice sites. Cystic kidneys were diagnosed in all fetuses, while all but two sibs had the ductal plate malformation of the liver. Polydactyly was never recorded. Clinical variability was observed with respect to CNS malformations, with encephalocele, Dandy-Walker malformation and cerebellar vermis hypo/aplasia being variably present. Genitalia abnormalities (including bicornate uterus or vaginal septum), and tetralogy of Fallot were recorded in single patients.

DISCUSSION

Here we present the results of the largest *TMEM67* screening so far reported in JSRD/MKS, leading to the identification of twenty novel mutations. In the JSRD group, we found mutations in the great majority of patients with liver involvement, ranging from the full-blown COACH syndrome phenotype (with proven hepatic fibrosis at liver biopsy) to milder presentations variably including hepatomegaly, elevation of liver enzymes and abnormal liver imaging. Along with our previously reported cases (Brancati et al., 2009) and with those published by Doherty and coworkers (Doherty et al., 2009), this brings the *TMEM67* mutation frequency in JS with liver involvement to 70% (32 out of 46), representing to date the strongest gene-phenotype correlate among JSRD subgroups (Valente et al., 2008).

The proportion of MKS fetuses mutated in *TMEM67* is much lower. We identified mutations in 12 out of 76 MKS probands, bringing the overall mutation frequency to 13% (35 out of 271 tested fetuses) (Baala et al., 2007; Consugar et al., 2007; Khaddour et al., 2007; Smith et al., 2006; Tallila et al., 2009). Yet, *TMEM67* remains to date one of the most commonly mutated gene in MKS, along with *MKS1* and *CC2D2A*. Yet, at difference from

TMEM67, both these genes present mutational hotspots explaining a large proportion of mutated cases (Tallila et al., 2009).

As previously reported, we observed that the feature most strikingly associated with *TMEM67* mutations, and in fact shared by all mutated patients regardless of other symptoms, is congenital liver involvement. In fact, Otto and collaborators reported *TMEM67* mutations in 8% NPH probands with CHF, but failed to identify mutations in 105 NPH patients lacking liver disease. Similarly, the two *TMEM67*-mutated families with ARPKD-like syndrome also presented CHF (Gunay-Aygun et al., 2009; Otto et al., 2009). This congenital liver disease originates by a developmental defect, the ductal plate malformation, resulting from failed remodeling of the ductal plate, that leads to bile duct proliferation and in many cases to overt CHF (Desmet, 1992). Defects in cilia formation and abnormal hepatic cell differentiation have been observed in the liver of some MKS fetuses (Clotman et al., 2008). Indeed, meckelin is known to be required for the correct cilium assembly, and it was also suggested to play a role in signal transduction at developmental stages preceding cilia formation in the liver (Clotman et al., 2008; Dawe et al., 2007). However, it is interesting to note that neither the rat (wpk) nor the mouse (bpck) models of MKS3 present any sign of liver involvement, while being affected by a rapidly progressive renal, retinal and neurological phenotype (Cook et al., 2009; Tammachote et al., 2009). Thus, the essential role of meckelin in liver development appears to be restricted to the human species, possibly representing a recently acquired function during the evolutionary process.

We failed to identify *TMEM67* mutations in any of 255 JSRD patients belonging to the other clinical subgroups, arguing against a major role of the gene in these phenotypes. In fact, out of about 600 JSRD families tested so far, only five with *TMEM67* mutations were reported that did not show any sign of hepatic involvement, confirmed by either long-term follow-up or appropriate liver investigations in four of them (Baala et al., 2007; Doherty et al., 2009; Otto et al., 2009). These patients may share yet unknown protective factors against the development of congenital liver disease, although the possibility that they harbor a peculiar combination of *TMEM67* alleles giving rise to a more benign phenotype is also possible. Of note, the allelic combinations are unique to these five families, and two of them share a substitution of the same proline residue in position 82 (Doherty et al., 2009). Functional studies in different cell types will help establish the different tissue-specific pathogenicity of such mutations.

In the present work, we report the identification of 20 novel mutations, raising to 87 the number of distinct pathogenic *TMEM67* changes so far reported (Baala et al., 2007; Brancati et al., 2009; Consugar et al., 2007; Doherty et al., 2009; Gunay-Aygun et al., 2009; Khaddour et al., 2007; Otto et al., 2009; Smith et al., 2006; Tallila et al., 2009). Of these, 19 recurred in two or more families while the others were reported in single cases. Overall, mutations are scattered throughout the entire gene with only two exons spared (exon 4 and 28). Mutations within eight out of 28 exons (2, 6, 8, 11, 13, 15, 18, 24) captured more than 60% of the *TMEM67* allelic spectrum, suggesting that these exons should be prioritized when performing molecular analysis (Figure 2).

Interestingly, a differential distribution of mutations along the gene in lethal (MKS) *versus* non-lethal (JSRD, NPH or ARPKD-like) phenotypes can be observed, in particular when considering missense mutations. In fact, in MKS patients most missense mutations (18/24, 75%) cluster within exons 8 to 15, encoding the extra-cellular region of meckelin that follows the cystein-rich domain. Conversely, in non lethal-phenotypes only one third of missense mutations are found within exons 8-15 (21/61, 34%; p=0.001) being sparse along the entire coding sequence of the gene (Figure 3).

In line with these findings, we also observed different combinations of mutation types between lethal and non-lethal phenotypes (Figure 4). In order to assess the statistical significance of this observation, we decided to exclude splice site mutations (which effect on protein function is difficult to predict) and considered only 23 lethal versus 33 non-lethal cases carrying either combination of truncating and missense mutations (Figure 4, left side). Two truncating mutations were observed in about one third of MKS patients (8/23, 35%) but in none of the patients with non-lethal phenotypes (p=0.001). A combination of a truncating with a missense mutation was detected in a similar proportion of lethal and non-lethal cases (6/23 *vs* 9/33, 26% *vs* 28%), yet the position of the missense mutation in the gene varied significantly in the two groups, falling within exons 8-15 in 66% of MKS cases, but never in non-lethal cases (p=0.01). Homozygous or compound heterozygous missense mutations were detected in 9 (39%) lethal and 24 (73%) non-lethal families respectively (p=0.045). At least one missense mutation affecting exons 8 to 15 was detected in all 9 MKS and in 17/24 (71%) of non-lethal cases, although this difference did not reach statistical significance.

In line with these findings, we also observed different combinations of mutation types between lethal and non-lethal phenotypes (Figure 4). In order to assess the statistical significance of this observation, we decided to exclude splice site mutations (which effect on protein function is difficult to predict) and considered only 23 lethal versus 33 non-lethal cases carrying either combination of truncating and missense mutations (Figure 4, left side). Two truncating mutations were observed in about one third of MKS patients (8/23, 35%) but in none of the patients with non-lethal phenotypes (p=0.001). A combination of a truncating with a missense mutation was detected in a similar proportion of lethal and non-lethal cases (6/23 *vs* 9/33, 26% *vs* 28%), yet the position of the missense mutation in the gene varied significantly in the two groups, falling within exons 8-15 in 66% of MKS cases, but never in non-lethal cases (p=0.01). Homozygous or compound heterozygous missense mutations were detected in 9 (39%) lethal and 24 (73%) non-lethal families respectively (p=0.045). At least one missense mutation affecting exons 8 to 15 was detected in all 9 MKS and in 17/24 (71%) of non-lethal cases, although this difference did not reach statistical significance.

Taken together, these findings suggest that missense mutations in exons 8 to 15 might have a more disruptive effect on the protein's function. One possibility is that a relevant subset of these mutations could affect splicing either by creating or disrupting splice sites or by affecting exonic splice enhancers/silencers, as already shown for other *TMEM67* coding variants (Baala et al., 2006; Kaddhour et al., 2007). Yet, we could not detect any significant differences between mutations falling within and outside exons 8 to 15 on their *in silico* predicted effects on splicing mechanisms. An alternative possibility is that the extracellular region encoded by exons 8 to 15 plays an crucial role for the protein's function, thus explaining the increased pathogenicity of missense mutations altering the aminoacidic structure of this region. To address this hypothesis, we have obtained PolyPhen PSIC scores for all reported missense mutations. Although the vast majority are predicted to be damaging, no significant differences were found in the frequency of benign, possibly and probably damaging changes falling within or outside exons 8-15, and the mean PSIC score obtained for this exon cluster was similar to that obtained for the remaining exons (data not shown). However, it must be noted that PolyPhen prediction is based on empirical rules applied to the amino acid sequence, phylogenetic profile scores, and calculation of structural parameters, and thus it is most accurate when the protein's structure is well characterized. Conversely, no information is available on the functional role of the region encoded by exons 8 to 15; moreover, we attempted to perform bioinformatic modelling of this region but could find no obvious close or even remote homologies with any known protein domain. This suggests a limited power in predicting pathogenicity of mutations affecting this region, and also implies a potential novel and unique role in meckelin function. Indeed, subcellular localization experiments showed that the c.1127A>C mutation within exon 11 (p.Q376P)

affected the ability of meckelin to localize at the cell surface, causing retention in the endoplasmic reticulum and consequent loss of function (Dawe et al., 2007). Detailed functional studies are needed to characterize the physiological function of this meckelin region and the disruptive effect of missense mutations.

The different pathogenic load of *TMEM67* mutations is also suggested by the fact that each combination of two given mutations is unique to a given ciliopathy, the only exception being represented by two families compound heterozygous for the two missense changes p.M252T and p.C615R, presenting with MKS and COACH syndrome respectively (Otto et al., 2009; Tallila et al., 2009). In this case, it is plausible to speculate that mutations or polymorphic variants located in other (ciliary) genes may play a relevant role as genetic phenotypic modifiers. This hypothesis, already put forward for variants in other ciliary genes (Khanna et al., 2009; Tory et al., 2007), opens novel enticing research perspectives to understand the increasing complexity of ciliary disorders.

Acknowledgments

We thank Dr Alessandro Ferraris for statistical support and Dr Emanuele Bellacchio for bioinformatic analysis; Antonio Lavelli, Viviana Speranza and Werner Rodriguez Acebo from Roche Applied Science for technical support; Mrs Karen Fontaine Marchand, Drs Clarisse Baumann Dieu-Coeslier, Virginia Kimonis, Laura Roos, Joelle Roume, Sabine Sigaudy and Prs Sylvie Manouvrier and Sylvie Odent for referring cases and genetic counseling. Contract grant sponsor: Italian Ministry of Health, MIUR, the March of Dimes, Burroughs Wellcome Fund NINDS, NIH. Contract grant numbers: Ricerca Corrente 2009 to BD; Ricerca Finalizzata 2006 ex art. 56 to EMV; Telethon grant n. GGP08145 to EB/EMV; NIH NS052455 and NS048453 grant to JGG; ANR grant N° MRAR2006/R06370KS to TAB. SZ is supported by INSERM-DGRSRT (CS/RN/2008 n°87).

Contract grant sponsor: Italian Ministry of Health, MIUR, the March of Dimes, Burroughs Wellcome Fund NINDS, NIH. Contract grant number: Ricerca Corrente 2009 (to BD); Ricerca Finalizzata 2006 ex art. 56 (to EMV); Telethon grant n. GGP08145 (to EB and EMV).

REFERENCES

Baala L, Romano S, Khaddour R, Saunier S, Smith UM, Audollent S, Ozilou C, Faivre L, Laurent N, Foliguet B, Munnich A, Lyonnet S, Salomon R, Encha-Razavi F, Gubler MC, Boddaert N, de LP, Johnson CA, Vekemans M, Antignac C, Attie-Bitach T. The Meckel-Gruber syndrome gene, MKS3, is mutated in Joubert syndrome. Am J Hum Genet. 2007; 80:186–194. [PubMed: 17160906]

Brancati F, Iannicelli M, Travaglini L, Mazzotta A, Bertini E, Boltshauser E, D'Arrigo S, Emma F, Fazzi E, Gallizzi R, Gentile M, Loncarevic D, Mejaski-Bosnjak V, Pantaleoni C, Rigoli L, Salpietro CD, Signorini S, Stringini GR, Verloes A, Zablocka D, The International JSRD Study Group. Dallapiccola B, Gleeson JG, Valente EM. MKS3/TMEM67 mutations are a major cause of COACH Syndrome, a Joubert Syndrome related disorder with liver involvement. Hum Mutat. 2009; 30:E432–E442. [PubMed: 19058225]

Clotman F, Libbrecht L, Killingsworth MC, Loo CC, Roskams T, Lemaigre FP. Lack of cilia and differentiation defects in the liver of human foetuses with the Meckel syndrome. Liver Int. 2008; 28:377–384. [PubMed: 17976156]

Consugar MB, Kubly VJ, Lager DJ, Hommerding CJ, Wong WC, Bakker E, Gattone VH, Torres VE, Breuning MH, Harris PC. Molecular diagnostics of Meckel-Gruber syndrome highlights phenotypic differences between MKS1 and MKS3. Hum Genet. 2007; 121:591–599. [PubMed: 17377820]

Cook SA, Collin GB, Bronson RT, Naggert JK, Liu DP, Akeson EC, Davisson MT. A mouse model for Meckel syndrome type 3. J Am Soc Nephrol. 2009; 20:753–764. [PubMed: 19211713]

Dawe HR, Smith UM, Cullinane AR, Gerrelli D, Cox P, Badano JL, Blair-Reid S, Sriram N, Katsanis N, ttie-Bitach T, Afford SC, Copp AJ, Kelly DA, Gull K, Johnson CA. The Meckel-Gruber Syndrome proteins MKS1 and meckelin interact and are required for primary cilium formation. Hum Mol Genet. 2007; 16:173–186. [PubMed: 17185389]

Desmet VJ. Congenital diseases of intrahepatic bile ducts: variations on the theme “ductal plate malformation”. Hepatology. 1992; 16:1069–1083. [PubMed: 1398487]

Doherty D, Parisi MA, Finn LS, Gunay-Aygun M, Al-Mateen M, Bates D, Clericuzio C, Demir H, Dorschner M, van Essen AJ, Gahl WA, Gentile M, Gorden NT, Hikida A, Knutzen D, Ozyurek H, Phelps I, Rosenthal P, Verloes A, Weigand H, Chance PF, Dobyns WB, Glass IA. Mutations in 3 genes (MKS3, CC2D2A and RPGRIP1L) cause COACH syndrome (Joubert syndrome with congenital hepatic fibrosis). J Med Genet. July 1.2009 epub.

Gerdes JM, Davis EE, Katsanis N. The Vertebrate Primary Cilium in Development, Homeostasis, and Disease. Cell. 2009; 137:32–45. [PubMed: 19345185]

Gunay-Aygun M, Parisi MA, Doherty D, Tuchman M, Tsilou E, Kleiner DE, Huizing M, Turkbey B, Choyke P, Guay-Woodford L, Heller T, Szymanska K, Johnson CA, Glass I, Gahl WA. MKS3-Related Ciliopathy with Features of Autosomal Recessive Polycystic Kidney Disease, Nephronophthisis, and Joubert Syndrome. J Pediatr. 2009; 155:386–92. [PubMed: 19540516]

Hildebrandt F, Zhou W. Nephronophthisis-associated ciliopathies. J Am Soc Nephrol. 2007; 18:1855–1871. [PubMed: 17513324]

Khaddour R, Smith U, Baala L, Martinovic J, Clavering D, Shaffiq R, Ozilou C, Cullinane A, Kyttala M, Shalev S, Audollent S, d'Humieres C, Kadhom N, Esculpavit C, Viot G, Boone C, Oien C, Encha-Razavi F, Batman PA, Bennett CP, Woods CG, Roume J, Lyonnet S, Genin E, Le MM, Munnich A, Gubler MC, Cox P, MacDonald F, Vekemans M, Johnson CA, ttie-Bitach T. Spectrum of MKS1 and MKS3 mutations in Meckel syndrome: a genotype-phenotype correlation. Mutation in brief #960. Online. Hum Mutat. 2007; 28:523–524. [PubMed: 17397051]

Khanna H, Davis EE, Murga-Zamalloa CA, Estrada-Cuzcano A, Lopez I, den Hollander AI, Zonneveld MN, Othman MI, Waseem N, Chakarova CF, Maubaret C, az-Font A, Macdonald I, Muzny DM, Wheeler DA, Morgan M, Lewis LR, Logan CV, Tan PL, Beer MA, Inglehearn CF, Lewis RA, Jacobson SG, Bergmann C, Beales PL, ttie-Bitach T, Johnson CA, Otto EA, Bhattacharya SS, Hildebrandt F, Gibbs RA, Koenekoop RK, Swaroop A, Katsanis N. A common allele in RPGRIP1L is a modifier of retinal degeneration in ciliopathies. Nat Genet. 2009; 41:739–45. [PubMed: 19430481]

Lancaster MA, Gleeson JG. The primary cilium as a cellular signaling center: lessons from disease. Curr Opin Genet Dev. 2009; 19:220–229. [PubMed: 19477114]

Marshall WF. The cell biological basis of ciliary disease. J Cell Biol. 2008; 180:17–21. [PubMed: 18180369]

Otto EA, Tory K, Attanasio M, Zhou W, Chaki M, Paruchuri Y, Wise EL, Utsch B, Wolf MT, Becker C, Nurnberg G, Nurnberg P, Nayir A, Saunier S, Antignac C, Hildebrandt F. Hypomorphic Mutations in Meckelin (MKS3/TMEM67) cause Nephronophthisis with Liver Fibrosis (NPHP11). J Med Genet. 2009; 46:663–670. [PubMed: 19508969]

Salonen R. The Meckel syndrome: clinicopathological findings in 67 patients. Am J Med Genet. 1984; 18:671–689. [PubMed: 6486167]

Salonen R, Paavola P. Meckel syndrome. J Med Genet. 1998; 35:497–501. [PubMed: 9643292]

Sharma N, Berbari NF, Yoder BK. Chapter 13 - Ciliary dysfunction in developmental abnormalities and diseases. Curr Top Dev Biol. 2008; 85:371–427. [PubMed: 19147012]

Smith UM, Consugar M, Tee LJ, McKee BM, Maina EN, Whelan S, Morgan NV, Goranson E, Gissen P, Lilliquist S, Aligianis IA, Ward CJ, Pasha S, Punyashthiti R, Malik SS, Batman PA, Bennett CP, Woods CG, McKeown C, Bucourt M, Miller CA, Cox P, Algazali L, Trembath RC, Torres VE, ttie-Bitach T, Kelly DA, Maher ER, Gattone VH, Harris PC, Johnson CA. The transmembrane protein meckelin (MKS3) is mutated in Meckel-Gruber syndrome and the wpk rat. Nat Genet. 2006; 38:191–196. [PubMed: 16415887]

Tallila J, Salonen R, Kohlschmidt N, Peltonen L, Kestila M. Mutation spectrum of Meckel syndrome genes: one group of syndromes or several distinct groups? Hum Mutat. 2009; 30:E813–E830. [PubMed: 19466712]

Tammachote R, Hommerding CJ, Sinders RM, Miller CA, Czarnecki PG, Leightner AC, Salisbury JL, Ward CJ, Torres VE, Gattone VH, Harris PC. Ciliary and centrosomal defects associated with mutation and depletion of the Meckel syndrome genes MKS1 and MKS3. Hum Mol Genet. 2009; 18:3311–23. [PubMed: 19515853]

Tory K, Lacoste T, Burglen L, Moriniere V, Boddaert N, Macher MA, Llanas B, Nivet H, Bensman A, Niaudet P, Antignac C, Salomon R, Saunier S. High NPHP1 and NPHP6 mutation rate in patients with Joubert syndrome and nephronophthisis: potential epistatic effect of NPHP6 and AHI1

mutations in patients with NPHP1 mutations. J Am Soc Nephrol. 2007; 18:1566–1575. [PubMed: 17409309]

Valente EM, Brancati F, Dallapiccola B. Genotypes and phenotypes of Joubert syndrome and related disorders. Eur J Med Genet. 2008; 51:1–23. [PubMed: 18164675]

Wittwer CT. High-resolution DNA melting analysis: advancements and limitations. Hum Mutat. 2009; 30:857–859. [PubMed: 19479960]

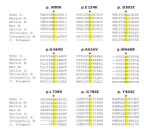

Figure 1.
Conservation across species of novel *TMEM67* missense mutations. *indicates affected residues

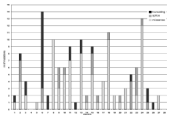

Figure 2.
Distribution across the *TMEM67* gene of truncating, splice site and missense mutations so far reported.

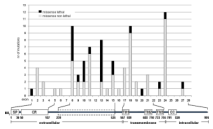

Figure 3.
Distribution across the *TMEM67* gene of missense mutations so far identified in patients with lethal (MKS) and non-lethal (JSRD, NPH and ARPDK-like) phenotypes. Correspondence between *TMEM67* exons and predicted meckelin domains is depicted at the bottom. SP: signal peptide; CR: cystein rich domain; TM1-2-3: transmembrane segments; CC: coiled coil domain; the dashed box indicates the extracellular region of meckelin encoded by exons 8 to 15.

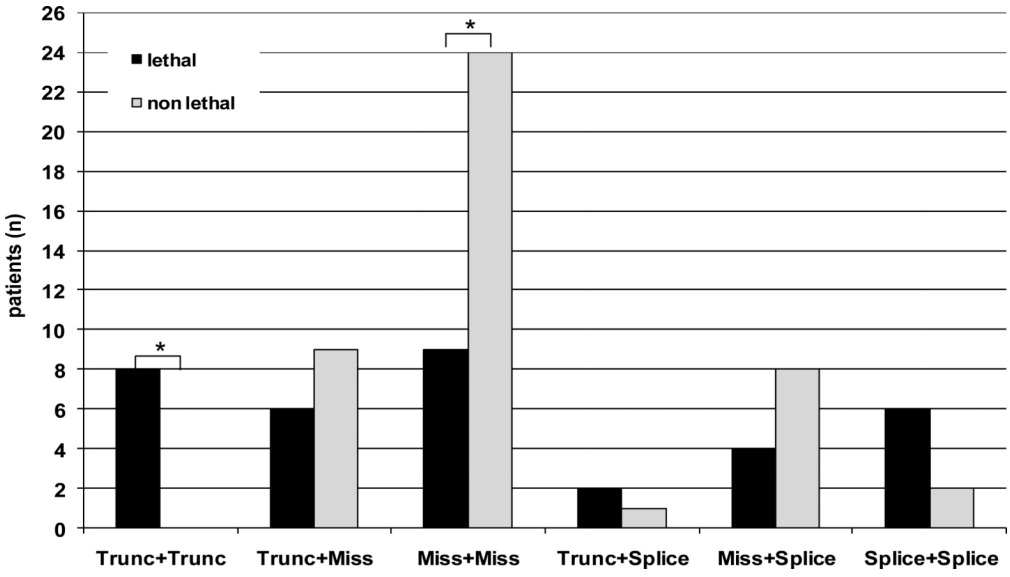

Figure 4.

Combination of mutation types so far reported in patients with lethal and non-lethal phenotypes. Statistical comparison has been performed only for the three groups on the left (two truncating mutations, truncating + missense, two missense mutations). Trunc: truncating (frameshift and nonsense mutations); miss: missense; splice: splice-site affecting mutation. *$p<0.05$

Table 1

Clinical and molecular data of eight JSRD families with biallelic *TMEM67* mutations

Family data				Clinical data					Genetic data			
Family		**Age (sex)**	**Origin**	**CNS**	**Eye**	**kidney**	**liver**	**other**	**Nucleotide changes**	**Exon**	**Effects on protein**	**Tr**
COR47	NG 1604	8(M)	IT	MT EV	Co	- (8)	ELE		c.370G>A	3	**p.E124K** +	P
	NG 2001	4(F)		MTS	ONH	- (4)	ELE		c.1073C>T	11	p.P358L +++	M
COR113	NG 2016	3(M)	IT	MTS	Co(L)	- (3)	LI		c.1706G>A	17	**p.G569D** ++	P
									c.G1860+1G>A	18	**Splice**	M
COR143	NG 2038	1(F)*	SW	MTS	Co	UCD	ELE		c.1285C>T	12	**p.Q429X**	M
									c.1847C>T	18	**p.A616V** +	P
COR212	NG 2358	15(F)	IT	MTS	Co	cysts, CRI (6m)	HSM ELE		c.1115C>A	11	p.T372K ++	M
									c.2216T>G	21	**p.L739R** ++	P
COR240	NG 2367	5(M)	IT	MTS	-	- (5)	HSM ELE LI		c.1077_1078	11	**p.T360RfsX18**	M
									c.1769T>C	17	p.F590S ++	P
COR265	NG 2511	23(M)	IT	MTS	Co(L)	- (23)	CHF	PD(4)	c.270T>G	2	**p.N90K** ++	M
									c.755T>C	8	p.M252T +++	P
COR266	NG 2515	4(F)	GE	MTS	Co	- (2)	CHF		c.300C>A	2	p.C100X	M
									c.2498T>C	24	p.I833T ++	P
COR254	NG 2466	8(M)	GE	MTS	-	- (8)	ELE		c.903C>G	9	**p.D301E** +	M
									c.1538A>G	15	p.Y513C +++	P

CHF: congenital hepatic fibrosis; Co: chorioretinal colobomas; CRI: chronic renal insufficiency; ELE: elevated liver enzymes; EV: enlarged ventricles; HSM: hepatosplenomegaly; IUGR: intrauterine growth retardation; (L): left eye only; LI: liver imaging consistent with ductal plate malformation; M: maternal; MTS: molar tooth sign; ONH: optic nerve hypoplasia; P: paternal; PD: polydactyly (number of limbs); Tr: transmission; UCD: urinary concentration defect. Origin: IT: Italy; SW: Sweden; GE: Germany.

*One pregnancy terminated for prenatal diagnosis of JSRD. PolyPhen prediction: benign:

+possibly damaging:

++probably damaging:

+++Novel mutations are in bold.

Table 2

Clinical and molecular data of 12 MKS families with biallelic *TMEM67* mutations

Family data				Clinical data					Genetic data			
Fam		**G.W.**	**Origin**	**CNS**	**eye**	**kidney**	**liver**	**other**	**Nucleotide changes**	**Exon**	**Effects on protein**	**Tr**
DEG	772	21(?)	US	Emc	n.a.	+	BDP		c.T2322+2dupT c.2561dupA	22 25	**Splice** **p.N854KfsX5**	M P
CHA	800	24(F)	FR	CVA	n.a.	+	BDP		c.1322G>T c.2002T>C	13 20	p.R441L +++ **p.W668R+++**	P M
LEF	812	26(F)	FR	Emc	n.a.	+	BDP	BU	c.2542G>T c.2357G>A	24 23	**p.E848X** **p.G786E** +++	M P
BAI	922 923	20(F) 17(F)	IT	DW CVA	n.a.	+ +	BDP BDP		c.675G>A c.2528A>G	7 24	p.W225X **p.Y843C** +++	M P
MAR	960	14 (?)	FR	Emc	n.a.	+	BDP	CP, IUGR	c.1046T>C c.2689_2690insTA	10 26	p. L349S ++ **p.L897IfsX64**	M P
DEB	965	19 (F)	FR	CVA H PMG	n.a.	+	BDP		c. T1413-1G>C c.2301delT	13 22	**Splice** **p.D768IfsX5**	M P
FOF	1007 1008	16(M) 13(M)	SN	DW DW	n.a.	+ +	- -		c.1538_1539delAT	15	**p.Y513X**	Ho
SAH	1044	26(F)	?	Emc DW	n.a.	+	BDP	VS	c.579delA	6	p.G195DfsX27	Ho
TAM	1077	13(F)	AL	Emc	n.a.	+	BDP	ToF	c.1046T>C	10	p.L349S ++	Ho
ELO	1088	?(M)	MO	Emc CVA	n.a.	+	BDP		c.579_580delAG	6	p.G195IfsX13	Ho

Hum Mutat. Author manuscript; available in PMC 2011 May 1.

Family data				Clinical data					Genetic data			
Fam		**G.W.**	**Origin**	**CNS**	**eye**	**kidney**	**liver**	**other**	**Nucleotide changes**	**Exon**	**Effects on protein**	**Tr**
COR 141	NG 2405	?(?)	FR	Emc	n.a.	+	BDP		c.1319G>A	13	p.R440Q ++	Ho
COR 238	NG 2357	21(?)	IT	Emc DW CCH	n.a.	+	BDP		c.387T>A	3	**p.C129X**	P
									c.755T>C	8	p.M252T +++	M

BDP: bile duct proliferation; BU: bicornate uterus; CCH: corpus callosum hypo/aplasia; CP: cleft palate; CVA: cerebellar vermis agenesis; DW: Dandy-Walker malformation; Emc: encephalomeningocele; G.W.: gestational week; H: hydrocephalus; Ho: homozygous; IUGR: intra-uterine growth retardation; n.a.: not available; PMG: polymicrogyria; ToF: tetralogy of Fallot; VS: vaginal septum. Origin: US: United States; FR: France; SN: Senegal; AL: Algeria; MO: Morocco. Other abbreviations as in Table 1.