

## CLINICAL STUDY

# Identification of novel mutations of the *WFS1* gene in Brazilian patients with Wolfram syndrome

Maria Regina R Gasparin, Felipe Crispim, Sílvia L Paula<sup>1</sup>, Maria Beatriz S Freire<sup>2</sup>, Ivaldir S Dalbosco<sup>3</sup>, Thais Della Manna<sup>4</sup>, João Eduardo N Salles<sup>5</sup>, Fábio Gasparin<sup>6</sup>, Aléxis Guedes, João M Marcantonio<sup>7</sup>, Márcio Gambini<sup>8</sup>, Camila P Salim and Regina S Moisés

Division of Endocrinology, Federal University of São Paulo, São Paulo, Brazil, <sup>1</sup>Federal University of Goiás, Goiânia, Brazil, <sup>2</sup>Medical School of Jundiaí, Jundiaí, Brazil, <sup>3</sup>University Foundation of Rio Grande, Rio Grande, Brazil, <sup>4</sup>Pediatric Endocrinology Unit, University of São Paulo, São Paulo, Brazil, <sup>5</sup>Medical School of Santa Casa de São Paulo, São Paulo, Brazil, <sup>6</sup>Department of Ophthalmology, University of São Paulo, São Paulo, Brazil, <sup>7</sup>Maringá, PR, Brazil and <sup>8</sup>Jundiaí, SP, Brazil

(Correspondence should be addressed to R S Moisés; Email: rmoises@unifesp.br)

## Abstract

**Objective:** Wolfram syndrome (WS) is a rare, progressive, neurodegenerative disorder with an autosomal recessive pattern of inheritance. The gene for WS, *WFS1*, was identified on chromosome 4p16 and most WS patients carry mutations in this gene. However, some studies have provided evidence for genetic heterogeneity and the genotype–phenotype relationships are not clear. Our aim was to ascertain the spectrum of *WFS1* mutations in Brazilian patients with WS and to examine the phenotype–genotype relationships in these patients.

**Design and methods:** Clinical characterization and analyses of the *WFS1* gene were performed in 27 Brazilian patients with WS from 19 families.

**Results:** We identified 15 different mutations in the *WFS1* gene in 26 patients, among which nine are novel. All mutations occurred in exon 8, except for one missense mutation which was located in exon 5. Although we did not find any clear phenotype–genotype relationship in patients with mutations in exon 8, the homozygous missense mutation in exon 5 was associated with a mild phenotype: onset of diabetes mellitus and optic atrophy during adulthood with good metabolic control being achieved with low doses of sulfonylurea.

**Conclusions:** Our data show that *WFS1* is the major gene involved in WS in Brazilian patients and most mutations are concentrated in exon 8. Also, our study increases the spectrum of *WFS1* mutations. Although no clear phenotype–genotype relationship was found for mutations in exon 8, a mild phenotype was associated with a homozygous missense mutation in exon 5.

*European Journal of Endocrinology* 160 309–316

## Introduction

Wolfram syndrome (WS) is a rare, progressive, neurodegenerative disorder with an autosomal recessive pattern of inheritance. The minimal diagnostic criteria for WS are non-autoimmune diabetes mellitus and optic atrophy; but other frequent findings are cranial diabetes insipidus, sensorineural deafness, urinary tract abnormalities, psychiatric illness, ataxia, peripheral neuropathy, and other endocrine disturbances.

The gene for WS, *WFS1*, was identified on chromosome 4p16 by two independent groups (1, 2). This gene consists of eight exons, spans 33.4 kb of genomic DNA and encodes an 890 amino acid protein named wolframin. Wolframin is a component of the unfolded protein response and has an important function in maintaining homeostasis in the endoplasmic reticulum in pancreatic  $\beta$ -cells (3). Since its identification, *WFS1*

gene mutations have been identified in most WS patients. However, some studies have provided evidence for genetic heterogeneity (4, 5). In fact, very recently Amr *et al.* showed that a mutation in a zinc-finger gene, *ZCD2*, also causes WS (6).

In the present study, we aimed to ascertain the spectrum of *WFS1* mutations in Brazilian WS patients and to examine the phenotype–genotype relationships in these patients.

## Subjects and methods

The study population comprised of 27 individuals (15 females and 12 males, aged 12–39 years old) from 19 unrelated families. The intra-family cases were all siblings. The criteria to diagnose WS were diabetes mellitus and bilateral optic atrophy. Patients were

recruited from different Brazilian regions with the main referral sources being university hospitals.

Patients were submitted to an interview to ascertain the family history and medical history of diabetes, visual loss, and associated manifestations. Detailed clinical examination was also performed. Standardized ophthalmologic evaluations with the pupils dilated by specialized ophthalmologists and ultrasonography of the urinary tract were performed in all participants. Diabetic nephropathy was assessed by 12 h urinary albumin excretion measurement and plasma creatinine concentrations. Audiological examination (pure-tone, speech, and impedance audiometry) was performed in 23 cases. Diabetes insipidus was investigated using the water deprivation test in patients whose urine specific gravity was less than 1010.

Free thyroxin (FT<sub>4</sub>), TSH, prolactin, cortisol, GH, testosterone (in postpubertal boys), estradiol (in postpubertal girls), LH, and FSH (in postpubertal patients) were measured in most patients. Brain magnetic resonance or computed tomography was performed in 12 patients.

Blood samples were obtained from each subject and genomic DNA was extracted from peripheral blood leukocytes using a commercial kit (Puregene DNA Isolation Kit, Gentra System, Minneapolis, MN, USA). Exons 2–8 and the intron–exon boundaries of the *WFS1* gene were amplified by PCR using 12 pairs of primers. The non-coding exon 1 was sequenced only when no mutation was found in the coding region. Exons 1–7 were amplified using a set of primers as previously published (7). Exon 8, which was divided into seven regions, was amplified using primer pairs as described by Strom *et al.* (2). The PCR products were directly sequenced using the Big Dye Terminator Cycle Sequencing Reaction Kit version 3.1 and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence changes of heterozygous frameshift mutations were determined by cloning the PCR product using TOPO TA Cloning (Invitrogen) and sequencing clones derived from both alleles.

The degree of functional impairment by novel amino acid substitutions was quantified using the Multivariate Analysis of Protein Polymorphism (MAPP) software (8). This method exploits the physicochemical variations of wild-type amino acid residues, missense variants and phylogenetic conservation. We used an alignment of six orthologues (*Homo sapiens*, *Macaca mulatta*, *Mus musculus*, *Rattus norvegicus*, *Xenopus tropicalis*, and *Drosophila melanogaster*).

The nomenclature recommended by the Human Genome Variation Society was used for the description of mutations. Numbering of nucleotides: the A of the ATG of the initiator Met codon was denoted as +1 (GenBank NM\_006005) and amino acids were numbered according to the GenBank accession number NP\_005996.1.

The presence of the 3243 A–G transition in the mitochondrial tRNA Leu (UUR) gene was determined by PCR, Apa I digestion, and acrylamide gel electrophoresis as previously reported (9). Bands were visualized by ethidium bromide staining. In this diagnostic system, the lower limit of detection for A3243G heteroplasmy is 1.0% (9). This procedure was performed in patients in whom no mutation was detected in *WFS1*.

One hundred chromosomes from 50 unrelated non-diabetic individuals (fasting plasma glucose < 100 mg/dl) were used as controls.

### Statistical analysis

All data are shown as means  $\pm$  s.d. Differences in continuous variables between the groups were evaluated by the unpaired Student's *t*-test. Differences between types of mutation and age at onset of diabetes were tested by ANOVA. A *P* < 0.05 was considered statistically significant.

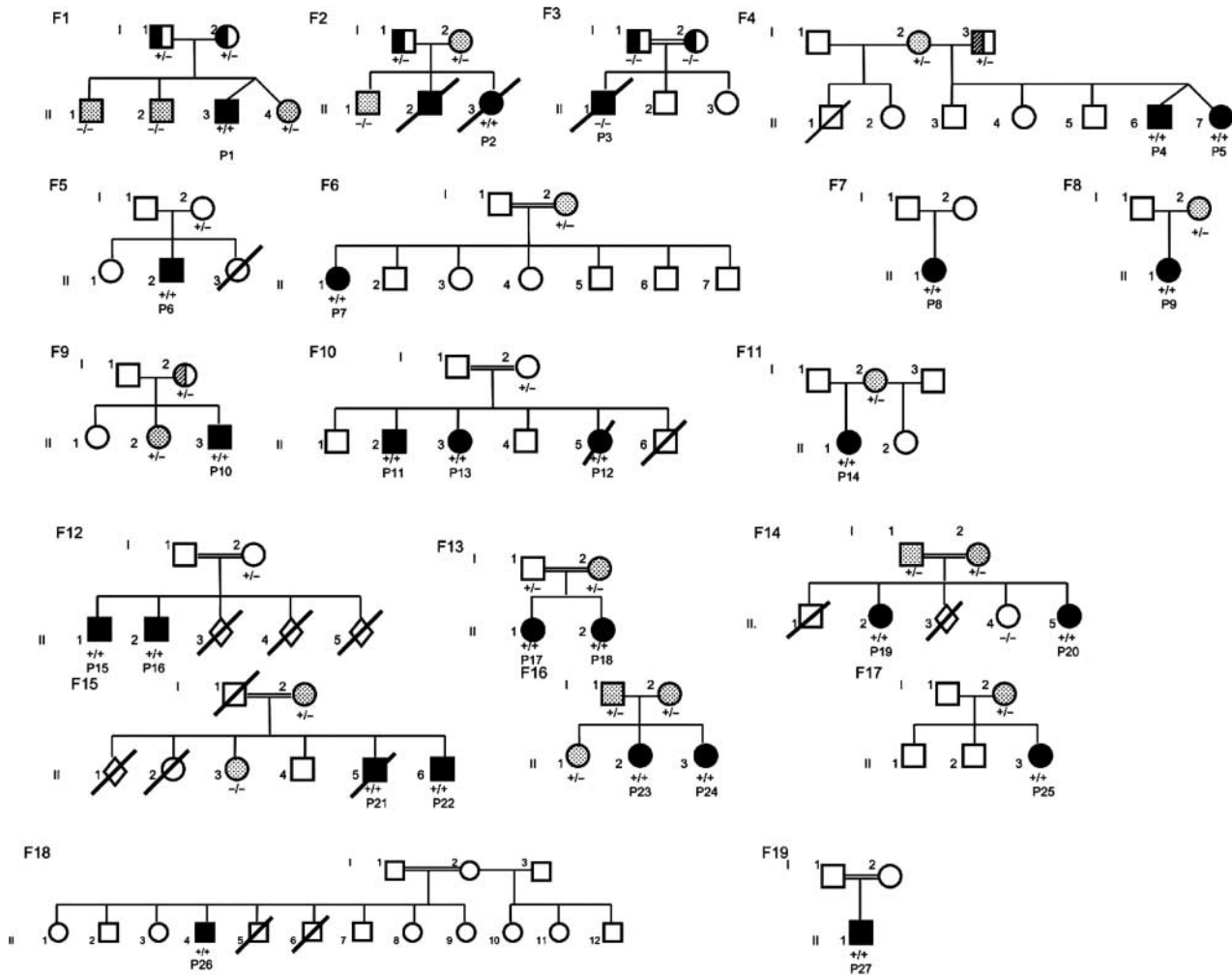
This study was approved by the Research Ethics Committee of the Paulista Medical School, Federal University of São Paulo, and all participants were informed about the aims of the study and gave their written consent.

## Results

### Clinical features

Twenty-seven patients (P1–P27) from 19 unrelated families (F1–F19) were studied. They were from the five Brazilian regions: north (F14), northeast (F13, F18), central-west (F5, F10), southeast (F1–F4, F6, F7, F11, F12, F15, F19), and south (F8, F9, F16, F17). Consanguinity was reported in nine families (47.4%) and one proband was an adopted girl with unknown family history. Pedigrees of the families are shown in Fig. 1 and the main clinical features of the affected individuals are shown in Table 1.

Diabetes was diagnosed at a mean age of  $6.7 \pm 5.0$  years old (range: 1.5–27 years) and for 25 patients (92.6%) it was the first manifestation of WS. In 24 cases (88.9%), diabetes was diagnosed in the first decade of life and in only one patient in the third decade. There was no significant difference in the age at onset of diabetes between siblings (*P* = 0.35). All patients, except one (P26), were treated with insulin from the onset. Diabetic retinopathy (non-proliferative and proliferative) was present in 5 out of the 27 patients (18.5%) all of whom had suffered from diabetes for more than 15 years. However, the mean duration of diabetes was not different in patients with and without retinopathy ( $18.2 \pm 2.95$  years vs  $17.2 \pm 5.10$  years, *P* = 0.66). Overt nephropathy was observed in two patients, one of whom had end-stage renal failure requiring hemodialysis. Both had proliferative retinopathy.



**Figure 1** Pedigrees of Wolfram syndrome families. +, presence of *WFS1* mutation; -, absence of *WFS1* mutation; ■ Wolfram syndrome; ◼ Diabetes mellitus; ◼ Impaired glucose tolerance; □ Glucose tolerance status not determined; ◻ Normal glucose tolerance.

Optic atrophy was diagnosed at mean age of  $11.8 \pm 3.8$  years (range: 7–20 years) and all patients had abnormal visual acuity.

Twenty-three patients were submitted to audiography with abnormal results in all cases. In spite of this, seven patients had no clinical evidence of hearing loss, while 11 required hearing aids. Urinary tract abnormalities were observed in 25 patients (92.6%). The findings of the ultrasonography examinations included incomplete bladder emptying, increased bladder capacity, trabeculated bladder, mild to severe bilateral pyelocaliceal ectasia and mild to severe bilateral hydroureteronephrosis. Thirteen patients (48%) needed intermittent bladder catheterization.

Diabetes insipidus was present in 11 (40.7%) patients. All were controlled with intranasal anti-diuretic hormones.

Four out of the twelve (33.3%) male patients had hypergonadotropic hypogonadism and 34% of the

female patients had irregular menstrual cycles and/or delayed menarche. The four cardinal components of WS (diabetes mellitus, diabetes insipidus, optic atrophy, and deafness) were found in 11 patients (40.7%).

**Mutations of the *WFS1* gene**

We identified 15 different mutations in 26 patients (26 out of the 27), among which nine are novel (Table 2). The novel mutations were: c.1355\_1370dup16 (p.Ala460HisfsX88); c.1234\_1237delGTCT (p.Val412-SerfsX29); c.1145T>C (p.Leu382Pro); c.876dupC (p.Leu293ProfsX13); c.1991T>C (p.Leu664Arg); c.2007T>G (p.Tyr669X); c.2643\_2646delCTTT (p.Phe882SerfsX69); c.2105G>A (p.Gly702Asp); c.472G>A (p.Glu158Lys). None of these novel mutations were found in the 100 control chromosomes, and within families they were only present as homozygous mutations in affected individuals.

**Table 1** Characteristics of the study population.

Family	Patient	Sex (M/F)	Age (years)	Consanguinity <sup>b</sup>	Age at diagnosis (years) <sup>a</sup>				Urinary tract abnormalities	Neurological abnormalities	Other complications
					DM	AO	DI	D			
F1	P1	M	27	—	3	12	3	12	17	Peripheral polyneuropathy; ataxia; cortical atrophy; mild sleep apnea; erectile dysfunction	Hypogonadism; mania
F2	P2	F	27 <sup>†</sup>	—	3	8	12	8	24	Mild mental retardation; cortical atrophy	Delayed puberty; hypothyroidism
F3	P3	M	31 <sup>†</sup>	+	14	14	—	+	+	Cortical atrophy	Renal failure; non-proliferative retinopathy
F4	P4	M	25	—	3.5	10	—	14	24	—	—
F4	P5	F	25	—	3.5	20	—	20	24	—	Hypertension; hypercholesterolemia
F5	P6	M	22	—	9	14	14	14	14	Nystagmus	—
F6	P7	F	19	+	6	15	18	12	17	—	Hypertension
F7	P8	F	14	—	6	11	10	14	+	Restless legs syndrome; peripheral neuropathy	Depression
F8	P9	F	27	—	8	8	—	NT	+	—	—
F9	P10	M	35	—	8	16	15	10	23	Erectile dysfunction	Hypogonadism; depression
F10	P11	M	27	+	9	9	24	19	+	—	Cataract
F10	P12	F	22	+	7	12	+	+	+	—	—
F10	P13	F	25 <sup>†</sup>	+	5	11	14	14	16	—	—
F11	P14	F	27	—	7	20	20	25	—	—	—
F12	P15	M	30	+	7	11	17	20	+	—	—
F12	P16	M	24	+	11	11	—	NT	+	—	—
F13	P17	F	21	+	3	9	—	19	14	—	Depression
F13	P18	F	18	+	3	10	—	10	9	—	Depression; anorexia non-proliferative retinopathy
F14	P19	F	25	+	2	15	—	23	20	—	Menarche 16.5 years severe proliferative retinopathy, neovascular glaucoma; diabetic nephropathy
F14	P20	F	13	+	4	9	—	11	9	—	—
F15	P21	M	26	+	8	13	—	26	13	Mild mental retardation	Hypogonadism; cataract; non-proliferative retinopathy; hypertelorism; low set posterior rotated ears
F15	P22	M	24 <sup>†</sup>	+	6	13	—	NT	17	Mild mental retardation	Hypogonadism; cataract; non-proliferative retinopathy hypertelorism; low set posterior rotated ears
F16	P23	F	23	—	8	7	—	18	—	—	Menarche 15 years
F16	P24	F	26	—	8	8	—	21	18	—	Menarche 15 years
F17	P25	F	19	—	2	7	—	14	7	Ataxia; polyneuropathy	Menarche 16 years glaucoma, cataract
F18	P26	M	39	+	27	19	—	39	33	Erectile dysfunction	—
F19	P27	M	12	+	1.5	7	—	NT	7	—	—

DM, diabetes mellitus; DI, diabetes insipidus; OA, optic atrophy; D, deafness.

<sup>a</sup>(+) Denotes complication present but the age at onset is unknown; (—) denotes complication absent; NT, not tested.<sup>b</sup>(+) Denotes consanguinity present; (—) denotes consanguinity absent.<sup>†</sup>Age of death.

**Table 2** Mutations in the *WFS1* gene.

Patients	Exon	Nucleotide change	Amino acid change	Type of mutation
P1	8	c.[1355_1370dup16]+ [1230_1233delCTCT]	p.[Ala460HisfsX88]+[Val412SerfsX29]	Frameshift; Frameshift
P2	8	c.[1941C>A]+[1949_1950delAT]	p.[Cys647X]+[Tyr650CysfsX61]	Nonsense; Frameshift
P4/P5	8	c.[1243_1245delGTC]+ [1234_1237delGTCT]	p.[Val415del]+[Val412SerfsX29]	Deletion; Frameshift
P6	8	c.[1230_1233delCTCT]+ [1230_1233delCTCT]	p.[Val412SerfsX29]+[Val412SerfsX29]	Frameshift; Frameshift
P7	8	c.[1243_1245delGTC]+ [1243_1245delGTC]	p.[Val415del]+[Val415del]	Deletion; Deletion
P8	8	c.[1145T>C]+[1230_1233delCTCT]	p.[Leu382Pro]+[Val412SerfsX29]	Missense; Frameshift
P9	8	c.[1949_1950delAT]+ [1060_1062delCTT]	p.[Tyr650CysfsX61]+[Phe354del]	Frameshift; Deletion
P10	8	c.[1243_1245delGTC]+ [1230_1233delCTCT]	p.[Val415del]+[Val412SerfsX29]	Deletion; Frameshift
P11/P12/ P13	8	c.[1243_1245delGTC]+ [1243_1245delGTC]	p.[Val415del]+[Val415del]	Deletion; Deletion
P14	8	c.[1230_1233delCTCT]+ [1140_1163dup24]	p.[Val412SerfsX29]+ [Asn714_Asn721dup]	Frameshift; Insertion
P15/P16	8	c.[1949_1950delAT]+ [1949_1950delAT]	p.[Tyr650CysfsX61]+[Tyr650CysfsX61]	Frameshift; Frameshift
P17/P18	8	c.[876dupC]+[876dupC]	p.[Leu293ProfsX13]+[Leu293- ProfsX13]	Frameshift; Frameshift
P19/P20	8	c.[1991T>C]+[1991T>C]	p.[Leu664Arg]+[Leu664Arg]	Missense; Missense
P21/P22	8	c.[2007T>G]+[2007T>G]	p.[Tyr669X]+[Tyr669X]	Nonsense; Nonsense
P23/P24	8	c.[1230_1233delCTCT]+ [2643_2646delCTTT]	p.[Val412SerfsX29]+[Phe882SerfsX69]	Frameshift; Frameshift
P25	8	c.[1230_1233delCTCT]+[2105G>A]	p.[Val412SerfsX29]+[Gly702Asp]	Frameshift; Missense
P26	5	c.[472G>A]+[472G>A]	p.[Glu158Lys]+[Glu158Lys]	Missense; Missense
P27	8	c.[1230_1233delCTCT]+ [1230_1233delCTCT]	p.[Val412SerfsX29]+[Val412SerfsX29]	Frameshift; Frameshift

Novel mutations are in bold.

Furthermore, all novel missense variants (p.Glu158Lys, p.Leu 382Pro, p.Leu664Arg, and p.Gly702Asp) presented high MAPP scores (19.98, 36.32, 33.02, and 35.86 respectively), thus they are envisaged as deleterious to protein function.

The c.1355\_1370dup16 (p.Ala460HisfsX88) is a 16 bp duplication in exon 8 found as heterozygous in patient P1, as well as in his mother and twin sister. This mutation causes a frameshift which results in a premature stop codon truncating the protein at amino acid residue 541. As a result 40% of the wolframins is lacking.

The c.1234\_1237delGTCT (p.Val412SerfsX29) is a 4 bp deletion in exon 8 identified as heterozygous in siblings P4 and P5 and their mother. This frameshift mutation results in a premature stop codon, predicting a truncated protein of 441 amino acids (49.6% shorter than the wild-type wolframins) with only the hydrophilic amino terminus and three transmembrane domains.

The c.1145T>C (p.Leu382Pro) is a missense mutation in exon 8 causing the substitution of the amino acid leucine by proline. It was found as heterozygous in patient P8.

The c.876dupC (p.Leu293ProfsX13) is a single base duplication at the beginning of exon 8 causing a frameshift which results in an early stop codon. The predicted truncated protein lacks the entire

transmembrane domains and the hydrophilic carboxy tail. This mutation was identified as homozygous in siblings P17 and P18 and as heterozygous in their parents.

The c.1991T>C (p.Leu664Arg) is a missense mutation located in exon 8 where the amino acid arginine substitutes the amino acid leucine. It was identified in siblings P19 and P20 as homozygous, and in their parents as heterozygous.

The c.2007T>G (p.Tyr669X) is a nonsense mutation in exon 8 which predicts a truncated protein with only 669 residues. It was found as homozygous in siblings P21 and P22, and as heterozygous in their mother and a paternal aunt.

The c.2643\_2646delCTTT (p.Phe882SerfsX69) is a 4 bp deletion in exon 8 resulting in a frameshift and termination signal at nucleotide 2854. The predicted sequence of the mutant protein is 61 amino acids longer than the wild-type protein. This mutation was found as heterozygous in siblings P23 and P24, as well as in their father and older sister.

The c.2105G>A (p.Gly702Asp) is a missense mutation in exon 8, resulting in the substitution of glycine by aspartic acid at codon 702. It was found as heterozygous in patient P25 and it is probably paternally inherited, as the patient's mother harbors the second mutation found in P25.

The c.472G>A (p.Glu158Lys) is a missense mutation in exon 5, which causes the substitution of glutamic acid by lysine at codon 158. This mutation was found in homozygous state in patient P26. DNA samples from his parents were not available, but consanguinity was reported.

The nonsense mutation p.Cys647X, found as heterozygous in patient P2, was previously reported in one Brazilian boy (10).

No mutations were detected in patient P3, who only carried five polymorphisms: (c.684G>C (p.=); c.997A>G (p.Ile333Val); c.1185T>C (p.=); c.1500T>C (p.=); c.1726G>A (p.Gly576Ser)) previously described (11). Complete cDNA sequencing was performed, including the non-coding exon 1. This patient, born of consanguineous parents, developed diabetes mellitus and optic atrophy at age 14 followed by a hearing deficit and urinary tract dilatation. The water deprivation test was performed and a normal response was obtained. The presence of the A3243G mutation in the tRNA Leu (UUR) gene was investigated but no mutation was found. A detailed description of this patient has previously been reported (12).

Causative mutations were homozygous in 14 cases (53.8%) and compound heterozygous in the other 12 cases. All mutations occurred in exon 8, except for one missense mutation located in exon 5, which was identified in patient P26. This patient showed a mild phenotype with optic atrophy and diabetes mellitus developing at ages 19 and 27 respectively. Since the diagnosis of diabetes 12 years ago, he has been treated with low-dose sulfonylurea tablets showing good metabolic control.

The most frequent mutation in this study was c.1230\_1233delCTCT (p.Val412SerfsX29) identified in eight families, corresponding to 44.4% of the pedigrees with *WFS1* mutations. This mutation causes a frameshift which results in a stop codon truncating the protein at residue 441. As a consequence, 49.5% of the wolframin is lost, including the majority of transmembrane domains and the entire hydrophilic carboxy tail.

Also, in our patient series various polymorphisms, composed of silent variants or conservative amino acid changes, were detected, all of which have previously been reported (13–17).

### Phenotype–genotype relationships

Possible phenotype–genotype relationships were investigated. The age at onset of diabetes mellitus was chosen as an indicator of disease severity. Missense mutations and 3 bp deletions, resulting in a deletion of one amino acid were considered non-inactivating mutations. Nonsense, frameshift mutations, deletions, and insertions of more than 3 bp were considered inactivating mutations (11). Among the 26 patients with *WFS1* mutations, 13 carried two inactivating, six carried one inactivating and one non-inactivating and seven carried

two non-inactivating mutations. Their mean ages at onset of diabetes were  $5.9 \pm 2.9$  years;  $5.1 \pm 2.5$  years, and  $8.5 \pm 8.4$  years respectively ( $P = \text{ns}$ ). However, the only patient harboring mutations in both alleles outside exon 8 showed a mild phenotype: the onset of diabetes mellitus and optic atrophy was during adulthood and good metabolic control was achieved with low doses of sulfonylurea. A hearing deficit was not clinically evident and diabetes insipidus was absent.

### Discussion

Since the cloning of the *WFS1* gene, genetic screening of WS patients has been performed mostly in Caucasian individuals (7, 14, 15, 18). The Brazilian population is mostly of Portuguese origin, miscegenated with a variety of other populational groups including Italians, Spaniards, Japanese, Africans, and native Indians. Thus, the Brazilian population has diverse ethnicity. In this study, one of the largest WS series studied, we present a detailed clinical evaluation and genetic analysis of the *WFS1* gene in 27 Brazilian patients. These individuals are from various regions of the country and should be representative of WS patients in Brazil. *WFS1* was found to be the major gene in our WS population with 15 different mutations in 26 patients; only one patient (P3) had no mutation. This patient had a typical WS phenotype presenting with diabetes mellitus, optic atrophy, hearing loss, and urinary tract dilatation. The possibility of mutations in the promoter and regulatory regions of *WFS1* cannot be ruled out. Another condition that shares some clinical similarities with WS is maternally inherited diabetes and deafness associated with an A3243G mutation in the mitochondrial tRNA Leu (UUR) gene. However, we did not find A3243G mutation in P3. Recently, Amr *et al.* identified mutations in the *ZCD2* gene as a cause of WS in three large consanguineous Jordanian families (6). The affected individuals presented some particular features such as upper gastrointestinal ulceration with bleeding tendency secondary to a platelet aggregation defect and absence of diabetes insipidus (5, 6). Interestingly, similar to the patients reported in the Jordanian study, diabetes insipidus was absent in patient P3, but bleeding tendency was not observed. Further investigations will indicate if patient P3 harbors *ZCD2* gene mutations.

Identified mutations consisting of insertions, deletions, nonsense, and missense were concentrated in the largest exon, exon 8, similar to other reports (1, 2, 7, 15, 18). Besides exon 8, we found a novel homozygous missense mutation in exon 5 in one patient. Mutations in exon 5 are infrequent; only a few cases have been reported in prior publications (2, 7, 15, 18) with all of them being compound heterozygous with mutations in exon 8.

The most frequent mutation in this study was a 4 bp deletion resulting in a frameshift and premature stop codon (c.1230\_1233del CTCT (p.Val 412SerfsX29))

found in eight WS pedigrees. These families come from distinct regions of Brazil and are apparently unrelated. However, additional analysis would be needed to further evaluate a potential founder effect in these families. This mutation has previously been reported in French (14) and Italian pedigrees (7, 19). Prior studies have shown common mutations in certain groups, such as in Spanish and Italian populations (7, 19, 20), but not in others (14).

We identified nine novel mutations in our series. They were presented as homozygous in four pedigrees, all with reported consanguinity. These mutations are likely to be pathogenic since they were absent in 100 control chromosomes, occur in evolutionarily conserved residues and were present as homozygous only in affected subjects.

In our study most patients presented with diabetes mellitus within the first decade of life (median age: 6 years) followed by optic atrophy (median age: 11 years). These findings are in agreement with previously reported series, particularly a large UK nationwide study of 45 patients where diabetes mellitus presented at a median age of 6 years old and optic atrophy at 10 years old (21).

Diabetes insipidus was found in 40.7% of our patients and the median age of diagnosis was 14.5 years (range 3–24 years). This proportion is lower than some prior reports (21–23) but not others (2, 24). Medlej *et al.* studying the Lebanese population, performed water deprivation tests in all patients and found partial or complete diabetes insipidus in 87% of cases, with median age of onset of polyuria at 9 years (range 4–26 years) (23). Since in our series the water deprivation test was not performed in all patients, we cannot exclude the possibility of some undiagnosed cases. Urinary tract abnormalities were present in nearly all of our patients, as described in other published series (22). However, lower prevalences have also been reported (21, 25), indicating clinical heterogeneity. The urinary tract abnormalities associated with WS are likely to be due to degeneration of innervation of the ureters and bladder. It is unclear whether the high urine output associated with diabetes mellitus and diabetes insipidus contribute to renal tract dilatation. Page *et al.* reported an improvement in dilatation with the treatment of diabetes insipidus (26).

Audiography showed abnormalities in 100% of the patients in whom it was performed, although seven patients were asymptomatic. The prevalence of hearing loss or abnormal audiography has been reported to be between 39 and 100% (21, 22, 27); probably representing differences in ascertainment. The audiological defect seen in these patients is a progressive bilateral neuro-sensorial hearing loss that first affects high frequencies.

The clinical data obtained were analyzed for phenotype–genotype correlation. As previously stated by Cryns *et al.* (11), mutations are described as non-inactivating or inactivating based on the type of mutation since no

functional studies are available to date. The age at onset of diabetes mellitus was chosen as an indicator of disease severity as it is much easier to detect diabetes early; while for the other components of WS, such as diabetes insipidus, and audiological and neurological findings, the patients may not notice the symptoms and so their presence may be underestimated. Considering mutations in exon 8, we found no significant difference in the mean age at onset of diabetes and the type of mutation. This lack of relationship is exemplified in family 14 where the affected individuals are homozygous for a missense mutation and the ages at onset of diabetes were very early (2 and 4 years). Similarly, Hardy *et al.* in a national UK cohort, found no clear correlation between any of the observed mutations and disease severity (15). However, Cano *et al.* studying French patients and combining his results with those of five published studies, including the data of Hardy *et al.* found that the presence of two inactivating mutations predisposed patients to an earlier age of onset of both diabetes mellitus and optic atrophy (24). On the other hand, we observed a milder phenotype in a patient carrying a homozygous missense mutation in exon 5. He is the only patient in our series who developed diabetes mellitus as an adult and has received long-term treatment with oral hypoglycemic drugs. Delayed expression of WS has been previously reported. Giuliano *et al.* noted an atypical phenotype in a patient whose *WFS1* coding region had only one mutation identified (14). In addition, a rare form of autosomal dominant transmission of diabetes mellitus, deafness, optic atrophy, and no other manifestations of Wolfram disease was associated with the E864K mutation in two families (28, 29). Thus, the location of *WFS1* mutations also seems important to determine the severity of disease. Future studies will determine which parts of wolframin are important for biological function.

In summary, here we present the first molecular characterization of Brazilian WS patients. Our data show that *WFS1* is the most common gene involved in WS of Brazilian patients with most mutations being concentrated in exon 8. Also, our study increases the spectrum of *WFS1* mutations. A common 4 bp deletion of nucleotides 1230–1233 was identified, indicating that screening studies in our population should begin with this region. Although no clear phenotype–genotype relationship was found in exon 8, a mild phenotype was associated with a homozygous missense mutation in exon 5.

## Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## Funding

This study was supported by a grant from the State of São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, grant # 05/01590-0).

## Acknowledgements

The authors are grateful to Magnus R Dias da Silva and Gustavo Guimarães for their help in MAPP analysis. We also thank the families for their participation in the study.

## References

- Inoue H, Tanizawa Y, Wasson J, Behn P, Kalidas K, Bernal-Mizrachi E, Mueckler M, Marshall H, Donis-Keller H, Crock P, Rogers D, Mikuni M, Kumashiro H, Higashi K, Sobue G, Oka Y & Permutt MA. A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). *Nature Genetics* 1998 **20** 143–148.
- Strom TM, Hortnagel K, Hofmann S, Gekeler F, Scharfe C, Rabl W, Gerbitz KD & Meitinger T. Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) caused by mutations in a novel gene (wolframin), coding for a predicted transmembrane protein. *Human Molecular Genetics* 1998 **7** 2021–2028.
- Fonseca SG, Fukuma M, Lipson KL, Nguyen LX, Allen JR, Oka Y & Urano F. WFS1 is a novel component of the unfolded protein response and maintains homeostasis of the endoplasmic reticulum in pancreatic  $\beta$  cells. *Journal of Biological Chemistry* 2005 **280** 39609–39615.
- Collier DA, Barrett TG, Curtis D, Macleod A, Arranz MJ, Maassen JA & Bunday S. Linkage of Wolfram syndrome to chromosome 4p16.1 and evidence for heterogeneity. *American Journal of Human Genetics* 1996 **59** 855–863.
- El-Shanti H, Lidral AC, Jarrah N, Druhan L & Ajlouni K. Homozygosity mapping identifies an additional locus for Wolfram syndrome on chromosome 4q. *American Journal of Human Genetics* 2000 **66** 1229–1236.
- Amr S, Heisey C, Zhang M, Xia XJ, Shows KH, Ajlouni K, Pandya A, Satin LS, El-Shanti H & Shiang R. A homozygous mutation in a novel zinc-finger protein, ERIS, is responsible for Wolfram syndrome 2. *American Journal of Human Genetics* 2007 **81** 673–683.
- Colosimo A, Guida V, Rigoli L, Di-Bella C, De-Luca A, Briuglia S, Stuppia L, Salpietro DC & Dallapiccola B. Molecular detection of novel WFS1 mutations in patients with Wolfram syndrome by a DHPLC-based assay. *Human Mutation* 2003 **21** 622–629.
- Stone EA & Sidow A. Physicochemical constraint violation by missense substitutions mediates impairment of protein function and disease severity. *Genome Research* 2005 **15** 978–986.
- Ohkubo K, Yamano A, Nagashima M, Mori Y, Anzai K, Akehi Y, Nomiyama R, Asano T, Urae A & Ono J. Mitochondrial gene mutations in the tRNA Leu(UUR) region and diabetes: prevalence and clinical phenotypes in Japan. *Clinical Chemistry* 2001 **47** 1641–1648.
- Zen PR, Pinto LL, Schwartz IV, Barrett TG & Paskulin G. Report of a Brazilian patient with Wolfram syndrome. *Journal of Pediatrics* 2002 **78** 529–532.
- Cryns K, Sivakumaran TA, Van den Ouweland JM, Pennings RJ, Cremers CW, Flothmann K, Young TL, Smith RJ, Lesperance MM & Van Camp G. Mutational spectrum of the WFS1 gene in Wolfram syndrome, nonsyndromic hearing impairment, diabetes mellitus, and psychiatric disease. *Human Mutation* 2003 **22** 275–287.
- Reis AF, Ferreira JG, Coifman R, Russo EMK, Moisés RCS & Dib S. Síndrome de Wolfram: Relato de um Caso. Revisão da Literatura e Caracterização do Diabetes Mellitus Associado. *Arquivos Brasileiros de Endocrinologia e Metabologia* 1996 **40** 106–112.
- Smith CJ, Crock PA, King BR, Meldrum CJ & Scott RJ. Phenotype-genotype correlations in a series of Wolfram syndrome families. *Diabetes Care* 2004 **27** 2003–2009.
- Giuliano F, Bannwarth S, Monnot S, Cano A, Chabrol B, Viallettes B, Delobel B, French Group of WS, & Paquis-Flucklinger V. Wolfram syndrome in French population: characterization of novel mutations and polymorphisms in the WFS1 gene. *Human Mutation* 2005 **25** 99–100.
- Hardy C, Khanim F, Torres R, Scott-Brown M, Seller A, Poulton J, Collier D, Kirk J, Polymeropoulos M, Latif F & Barrett T. Clinical and molecular genetic analysis of 19 Wolfram syndrome kindreds demonstrating a wide spectrum of mutations in WFS1. *American Journal of Human Genetics* 1999 **65** 1279–1290.
- Torres R, Leroy E, Hu X, Katrivanou A, Gourzis P, Papachatzopoulou A, Athanassiadou A, Beratis S, Collier D & Polymeropoulos MH. Mutation screening of the Wolfram syndrome gene in psychiatric patients. *Molecular Psychiatry* 2001 **6** 39–43.
- Kato T, Iwamoto K, Washizuka S, Moria K, Tajimab O, Akiyama T, Nankod S, Kunugi H & Katof N. No association of mutations and mRNA expression of WFS1/wolframin with bipolar disorder in humans. *Neuroscience Letters* 2003 **338** 21–24.
- van den Ouweland JM, Cryns K, Pennings RJ, Walraven I, Janssen GM, Maassen JA, Veldhuijzen BE, Arntzenius AB, Lindhout D, Cremers CW, Van Camp G & Dikkeschei LD. Molecular characterization of WFS1 in patients with Wolfram syndrome. *Journal of Molecular Diagnostics* 2003 **5** 88–95.
- Tessa A, Carbone I, Matteoli MC, Bruno C, Patrono C, Patera IP, De Luca F, Lorini R & Santorelli FM. Identification of novel WFS1 mutations in Italian children with Wolfram syndrome. *Human Mutation* 2001 **17** 348–349.
- Gomez-Zaera M, Strom TM, Rodriguez B, Estivill X, Meitinger T & Nunes V. Presence of a major WFS1 mutation in Spanish Wolfram syndrome pedigrees. *Molecular Genetics and Metabolism* 2001 **72** 72–81.
- Barrett TG, Bunday SE & Macleod AF. Neurodegeneration and diabetes: UK nationwide study of Wolfram (DIDMOAD) syndrome. *Lancet* 1995 **346** 1458–1463.
- Simsek E, Simsek T, Tekgul S, Hosal S, Seyrantepe V & Aktan G. Wolfram (DIDMOAD) syndrome: a multidisciplinary clinical study in nine Turkish patients and review of the literature. *Acta Paediatrica* 2003 **92** 55–61.
- Medlej R, Wasson J, Baz P, Azar S, Salti I, Loiselet J, Permutt A & Halaby G. Diabetes mellitus and optic atrophy: a study of Wolfram syndrome in the Lebanese population. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 1656–1661.
- Cano A, Rouzier C, Monnot S, Chabrol B, Conrath J, Lecomte P, Delobel B, Boileau P, Valero R, Procaccio V, Paquis-Flucklinger V, French Group of Wolfram Syndrome, & Viallettes B. Identification of novel mutations in WFS1 and genotype-genotype correlation in Wolfram syndrome. *American Journal of Medical Genetics. Part A* 2007 **143** 1605–1612.
- Kinsley BT, Swift M, Dumont RH & Swift RG. Morbidity and mortality in the Wolfram syndrome. *Diabetes Care* 1995 **18** 1566–1570.
- Page MM, Asmal AC & Edwards CR. Recessive inheritance of diabetes: the syndrome of diabetes insipidus, diabetes mellitus, optic atrophy and deafness. *Quarterly Journal of Medicine* 1976 **45** 505–520.
- Cremers CW, Wijdeveld PG & Pinckers AJ. Juvenile diabetes mellitus, optic atrophy, hearing loss, diabetes insipidus, atonia of the urinary tract and bladder, and other abnormalities (Wolfram syndrome). A review of 88 cases from the literature with personal observations on 3 new patients. *Acta Paediatrica Scandinavica* 1977 **264** 1–16.
- Eiberg H, Hansen L, Kjer B, Hansen T, Pedersen O, Bille M, Rosenberg T & Tranebjaerg L. Autosomal dominant optic atrophy associated with hearing impairment and impaired glucose regulation caused by a missense mutation in the WFS1 gene. *Journal of Medical Genetics* 2006 **43** 435–440.
- Valéro R, Bannwarth S, Roman S, Paquis-Flucklinger V & Viallettes B. Autosomal dominant transmission of diabetes and congenital hearing impairment secondary to a missense mutation in the WFS1 gene. *Diabetic Medicine* 2008 **25** 657–661.

Received 11 November 2008

Accepted 16 November 2008