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A case of a mild Wolfram Syndrome with concomitant *ATP7B* mutation

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Abstract

Background: **Wolfram Syndrome 1 (WS1) has been characterized on the basis of mutation in the WFS1 gene encoding a calcium storage wolframin endoplasmatic reticulum transmembrane glycoprotein.**

Case Presentation: **We observed a WS 10-years old female subject, with Type 1 diabetes-mellitus (DM), that had compound heterozygous WSF1 mutations but without other symptoms generally observed in WS subjects, such as optic atrophy or neurodegeneration.**

Results: **Decreased copper, ceruloplasmin, and transferrin levels, pointing to a copper deficiency, were associated with a new c.1870-3A>G mutation in the ATP7B gene, while lower calcium levels were associated with WSF1 mutations. An omega-3 fatty acids therapy was administrated to the subject in the attempt to ameliorate diabetes symptoms, restored copper deficiency, and normal calcium levels.**

Conclusions: **This specific case report provides new insights into the potential interplay of ATP7B mutation in shaping a milder WS clinical picture.**

Introduction

Wolfram syndrome (WS) is a rare disease[1], estimated to afflict about 1 in 770,000 in the UK and affecting about 1% of the world's population (https://www.orpha.net/consor/cgi-bin/index.php). According to the draft International Classification of Diseases (ICD-11), WS is categorized as a specified diabetes mellitus (DM; subcategory 5A16.1), and is also known as insipidus-diabetes mellitus-optic atrophy-deafness syndrome (DIDMOAD). Two types of WS have been characterized on the basis of mutations in different genes: WS1, with mutation in *WFS1* encoding for a calcium storage wolframin endoplasmatic reticulum (ER) transmembrane glycoprotein, and WS2, caused by *CISD2* mutations, which codes for a protein located in ER and mitochondria. WS is considered a prototype of ER disease[2-4]. Eukaryotic cells have a defense system called the "unfolded protein response", which protects cells from ER stress[5]. ER stress enhances *WFS1* expression, suggesting that *WFS1* mutations increase susceptibility to ER stress that leads to cell death and WS onset[6,7].

Case report

In this case study, a 10-year-old female subject (named here as 'Investigational Subject' ISj) was first admitted at the San Raffaele Hospital, Italy, with type 1 diabetes. ISj was screened for a number of Type 1 diabetes and WS genes and was found to be a compound heterozygous for c.316-1G> A and c.757 A>T mutations in the *WFS1* gene, classified as rare with uncertain significance and likely pathogenic. Based on these data, a diagnosis of WS1 was made. Unexpectedly, she had a mild WS1 symptomatology, showing no deficit in the optic nerve

or hearing problems, but characterized by type 1 diabetes (data not shown). The WS subject came to our attention for copper status evaluation, since emerging evidence[7] associated high levels of copper to type 1 diabetes.

ISj underwent a complete metabolomics analysis carried out by Metabolon (North Carolina, NC, USA; **Supplemental Tables 1 and 2**). This work revealed abnormalities in pathways that use copper as cofactor. Copper is an essential trace metal and a co-factor for a number of vital enzymes in metabolism involved in various metabolic pathways[6]. In particular, the results showed: (a) decreased levels of two metabolites linked to the polyamine cycle: 4-guanidino butanoate and beta-alanine; (b) decreased level of one metabolite linked to the amino acid tryptophan cycle: chinurenine; (c) decreased levels of two metabolites resulting from the catabolism of ascorbate: threonate and oxalate (Figures 1, 2. and 3). Therefore, ISj was screened for a panel of copper and associated metal biological variables, including measures of copper, iron, ceruloplasmin, transferrin, ferritin, percentage of transferrin saturation (% TfSat), ceruloplasmin-transferrin (Cp:Tf) antioxidant system as revealed by the Cp:Tf ratio (Table 1). While ferritin and iron were within the normal range, excluding iron deficiency, copper status levels were disturbed, with a copper deficiency typified by decreased copper (pediatric normal range 11.8-24.8 μmol/L[8]) and ceruloplasmin (Table 1). This result was unexpected since type 1 diabetes has been associated with a higher level of copper in general circulation[7]. The increase of the Cp:Tf ratio likely reflects processes of both oxidative stress and inflammation[9,10]. To investigate the copper deficiency associated to WS, ISj mother (Mo), ISj father (Fa), and three additional WS1 subjects (S1, S2, and S3) had copper panel evaluation (Table 1). While Fa and the three WS1 subjects showed normal to high levels, Mo had copper and ceruloplasmin levels lower than the normal range (Table 1). To further investigate copper deficiency, DNA from ISj and Mo blood was extracted and analyzed for the entire coding sequence of *ATP7B* gene. *ATP7B* mutations cause Wilson's disease (WD), an inherited metabolic disorder of impaired copper transport. Sequencing analysis showed in ISj a heterozygous condition for a new variant c.1870-3A>G and in Mo a compound heterozygosity for two variants: c.98T>C (p.M33T) and c.1870-3A>G (Figure 4).

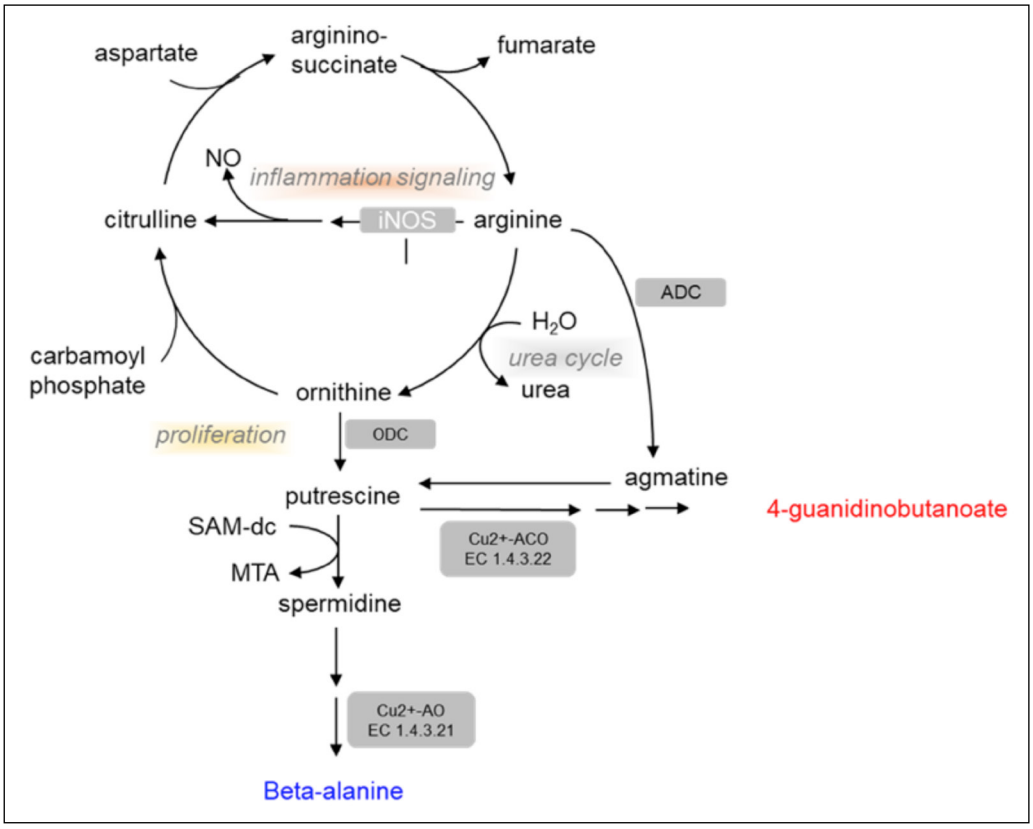

Figure 1. Polyamine cycle: role of copper enzymes

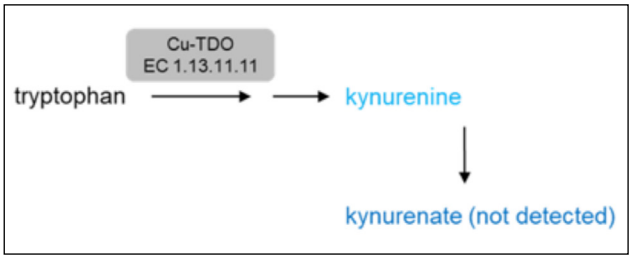

Figure 2. Metabolic way of tryptophan/kynurenine.

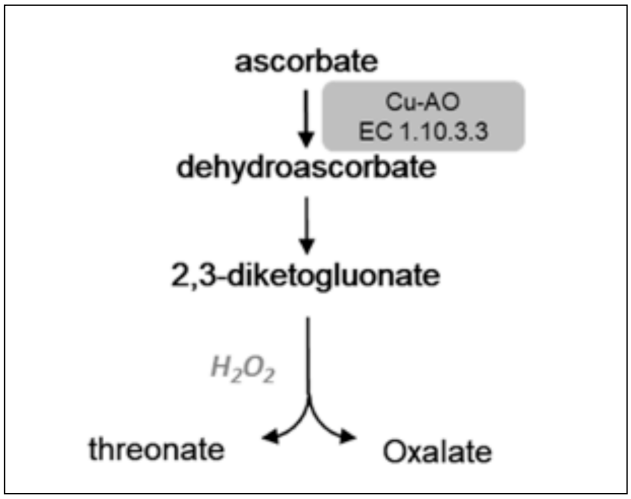

Figure 3. Metabolic way of ascorbate.

The new variant c.1870-3A>G was not detected in 300 control chromosomes, 1000 genomes Project database nor in ClinVar archive, and was then classified as rare with an "uncertain significance". *In silico* study (https://www.interactive-biosoftware.com/alamut-visual/) indicated that c.1870-3A>G mutation could affect the splicing site of the mRNA with a high probability of producing an incorrect mRNA maturation that is translated into a protein of altered size and shape (Figure 5).

To exclude the possibility of WD, the Kayser-Fleischer Ring examination was performed on both ISj and Mo, and it resulted negative. Mo also underwent a measure of 24 hours (24 h) urine copper concentrations in basal conditions and after 1000 mg/day of D-penicillamine (D-pen; "D-pen challenge test"). Mo's 24 h urine copper was 4 µg/day, within the normal range (40 µg/day; upper limit of normal reported as representative of a "normal" value of urine human copper excretion[11,12]). However, after the D-pen challenge test, the value raised to 291 µg/day, which is higher than 200 µg/24 h (5x ULN). According to Nicastro et al[11], this might be associated to asymptomatic WD subjects.

ISj began an omega-3 fatty acids (OM3FA) eicosapentaenoic acid (EPA) therapy to ameliorate the type 1 diabetes symptoms. After 3 months under OM3FA/EPA therapy, ISj's transferrin and bilirubin values remained lower, while copper and

Table 1. Copper and iron panel for individuals ISj, Mo, Fa, and the WS patients: S1, S2, and S3.

	ISj (age 10.3 years old)	ISj after 3 months OM3FAs Therapy	Mo (ISj mother)	Fa (ISj father)	S1 (WS subject)	S2 (WS subject)	S3 (WS subject)	Reference values (Pediatric)	Reference Values (Adult)
Copper (µmol/L)	**8.9**	**14.5**	**9.0**	12.9	12.8	18.7	11.7	*11.8-24.8	12.6-24.4 mmol/l (Fem) or 11.0-24.0 mmol/l (Male)
Ceruloplasmin (Cp) mg/dL	**17.4**	**28.9**	**18.9**	25.7	26.4	29.0	26.7	20-60 mg/dL	20-60 mg/dL
Iron µg/dL	73	-	-	-	66	47	65		40-160 µg/dL
Ferritin ng/mL	42.1	-	-	-	28.3	55.2	66.8		7-140 mg-dl
Transferrin mg/dL	192	-	-	-			325		200-360 mg/dl
Transferrin saturation	30%	-	-	-			16%		15-50%
Cp-Tf (Cp:Tf *0.01)	9.06	-	-	-			8.2		9.54-10.46[9]

*Pediatric reference intervals for serum copper and zinc.

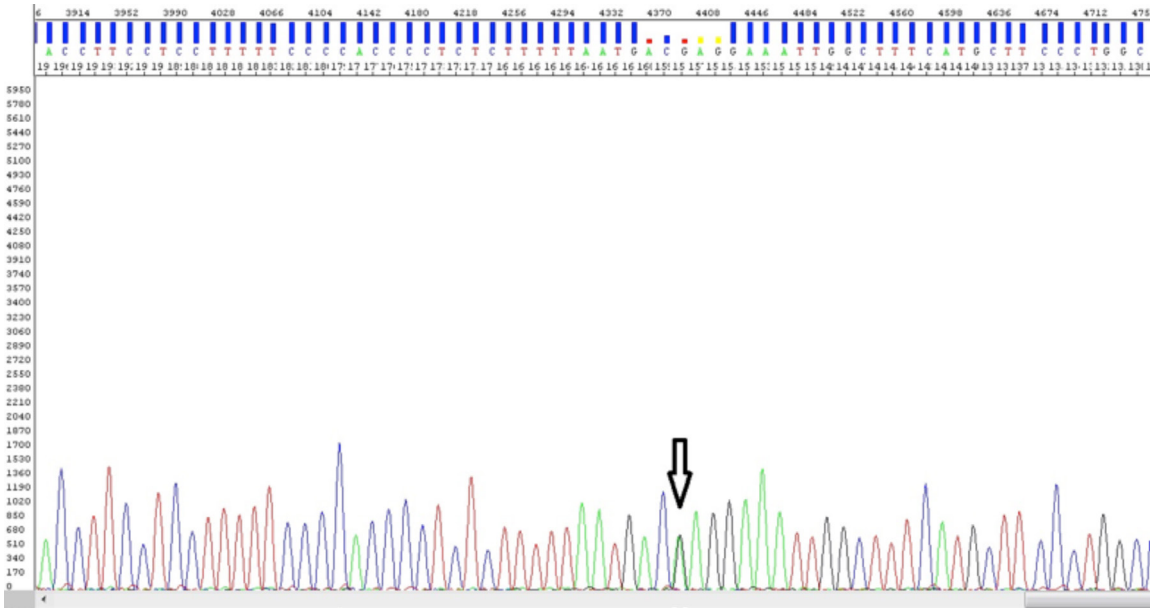

Figure 4. Chromatography from Sanger technique of the mutation c.1870-3A>G presented in ISj and Mo.

ceruloplasmin levels reached normal range, as shown in Table 1 (second column). In line with this finding, changes in the activity of the copper enzymes described above (data not shown) and other metabolites have been returned to normal levels (**Supplemental Table 3 and Supplemental Table 4**). Long-chain omega-3 fatty acids/EPA have been shown to have beneficial effects in the management

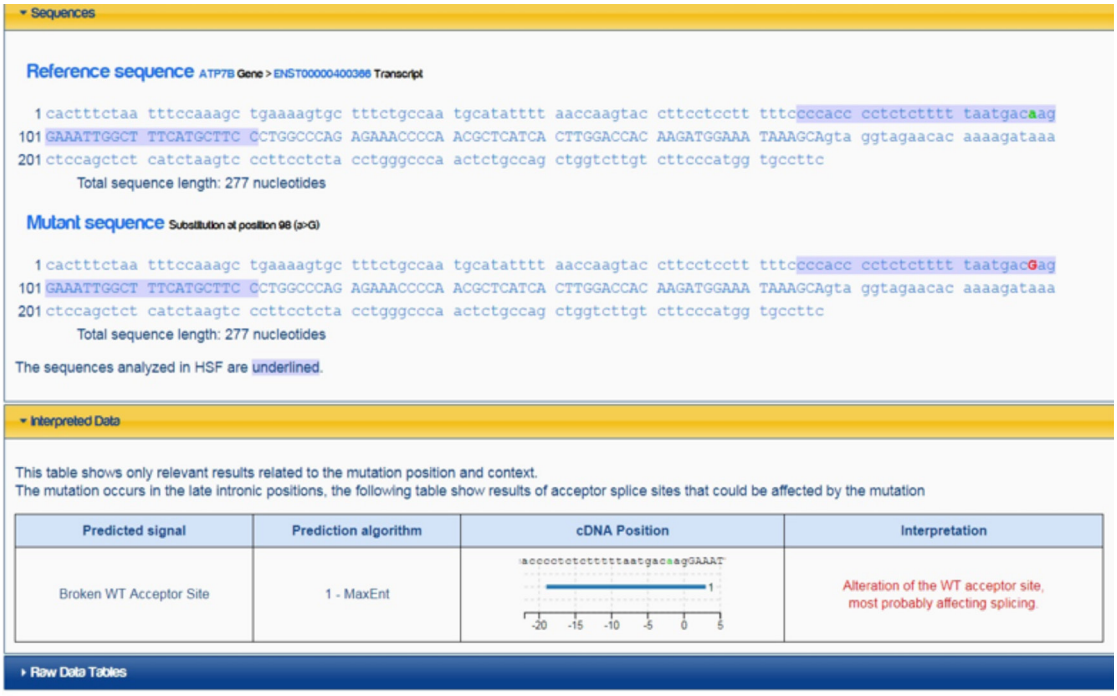

Figure 5. The figure shows the result of the pathogenicity prediction using the bioinformatics tool 'Alamut' (https://www.interactive-biosoftware.com/alamut-visual/). As indicated, the mutation could have the effect of altering the splicing site of the messenger RNA with a high probability of producing an incorrect maturation of the same messenger RNA that is translated into a protein of altered size and shape.

of various inflammatory and pro-oxidant states. The primary role of EPA is as an anti-inflammatory agent, and its appropriate use has been proven to be effective in both experimental and clinical trials in reducing concentrations of inflammatory markers, such as cytokines and leukotrienes, and have shown some effects on diabetes[13]. Docosahexaenoic acid (DHA) supplementation improves liver steatosis and insulin sensitivity in children with non-alcoholic fatty liver disease[14] , which has been associated with a mild copper deficiency[15]. Also, we can speculate potential beneficial effects of DHA on copper deficiency, in line with our observation.

Furthermore, we measured, through the inductively coupled plasma mass spectrometry (ICP-MS), the content of other metals and metalloid in the serum of subject ISj, Mo, and three subjects affected by WS (named S1, S2, and S3; Table 2). ISj's metal levels were measured before and after a three-month period of OM3FA therapy. Among the elements analyzed, calcium was decreased in all WS affected subjects (S1, S2, and S3) and ISj before OM3FA therapy), showing a shared molecular feature, as described in literature[1]. Calcium levels increased in subject ISj after treatment, approaching normal values (Table 2). It has been reported that wolframin protein binds to sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and modulates its function[16]. SERCA is a mammalian membrane-bound protein sustaining Ca^{2+} transport and involved in cell Ca^{2+} signaling and homeostasis[17]. The activation of SERCA can sustain high ER calcium levels even under pathological conditions that could prevent death of neurons and β cells[16,17]. WS1 mutations can affect wolframin -SERCA interaction, resulting in low level of calcium, in line with our observation of lower levels of calcium detected in the serum of WS subjects (Table 2), and in ISj before OM3FA/EPA therapy. Since ISj's calcium levels approached normal values after the OM3FA/EPA therapy, we can speculate that the treatment had some beneficial effects in maintaining SERCA activity. Vanadium was also consistently increased in both ISj and Mo (40 times higher than normal range), while levels were within the reference range in other WS subjects. The abnormalities found in Vanadium may be ascribed to the *ATP7B* c.1870-3A>G mutation or to a source of environmental contamination or exposure associated to ISj and Mo living area (Table 2).

The substitution of an adenosine (A) with a guanine (G) at the negative splice site of exon 6 (c.1870-3A>G) produces an alteration in splicing of mRNA, and results in an altered ATPase7B protein[18]. The presence of heterozygosity of the c.1870-3A> G mutation classifies ISj as healthy *ATP7B* carriers. This mutation can explain the levels below the normal values of copper and ceruloplasmin observed in ISj and in Mo. Table 3 shows the single nucleotide polymorphisms (SNPs) of the *ATP7B* gene identified in ISj and Mo. Of note, is the fact that Mo presents the missense variant p.M33T in exon 2. The VarSome-clinical platform indicated that the p.M33T mutation is rare [frequency of the least frequent allele in the normal population (Minor allele frequency) MAF <0.01] and classified as genetic variant "with uncertain significance". The fact that this mutation was not inherited by ISj is suggestive that the two mutations c.1870-3A>G and M33T lie on different chromosomes according to the Mendelian law of segregation of alleles. This suggests a high probability that Mo is a compound heterozygote for c.1870-3A>G / M33T, and that both *ATP7B* copies are altered. The clinical and biological meaning of the p.M33T variant remains unknown and further works are needed to reveal whether it has a clinical impact in WD onset. In an anamnestic interview, Mo reported that she suffered from anorexia at the age of 20 which could be suggestive of asymptomatic WD, although a diagnosis cannot be posed since she is in good health. While type I diabetes is associated with elevated levels of serum/plasma copper[7], ISj, diagnosed with type I diabetes, had lower than normal basal levels that normalized after a three months OM3FA/EPA therapy. Although speculative, it cannot be excluded that the c.1870-3A>G *ATP7B* mutation could have exerted modulation effects on WS1 mutation penetrance, or on the complications associated with diabetes type I, contributing to the relatively mild clinical picture exhibited by ISj. The *ATP7B* gene is a highly polymorphic gene, and its mutations or single SNPs can have very different effects in relation to the "biological context". For example, two SNPs K832R and R952K have recently been identified as risk factors for Alzheimer's disease[19-23], as modulators of levels of non-ceruloplasmin copper. Their effect seems to be cancelled by the c.1870-3A>G heterozygous mutation identified in ISj and Mo who shows no altered levels of non-ceruloplasmin copper (data not shown).

Table 2. ICP-MS multielement panel for individuals ISj, ISj after OM3FA therapy, Mo, and the WS subjects: S1, S2, and S3.

ID	Diagnosis	Co	V	Mn	Cr	As	Se	Zn	Fe	Ca	Mg	Cd	Pb	Hg	Al	Ni	Mo
S1	WS	0.22	<0.05	0.59	0.11	<0.5	63.8	699	709	**72328**	12415	<0.1	<0.1	<0.5	8.2	0.32	0.59
S2	WS	0.25	<0.05	1.83	0.15	<0.5	73.3	804	661	**86340**	17686	<0.1	<0.1	<0.5	20	0.48	1.65
S3	WS	0.25	<0.05	1.14	2.37	<0.5	100	982	1156	**94613**	19403	<0.1	<0.1	0.89	10.3	0.53	1.81
Mo	-	0.21	**4.44**	1.23	0.35	3.83	102	828	705	**99443**	22100	<0.1	<0.1	1.12	7.44	0.54	1.83
ISj	WS	<0.1	**4.22**	1.47	0.66	0.82	76.9	840	1012	**90199**	19508	<0.1	0.38	<0.5	6.19	0.61	2.83
Sj after OM3FA	WS	0.12	**3.99**	1.43	1.10	5.12	81.5	824	699	**99251**	19788	<0.1	<0.1	<0.5	5.56	0.55	2.87
Reference Values		<0.607	<0.115	<1.41	<0.294	<3.12						<0.27	<0.6	<1.89	<10	<0.9	<1.83
Minimal							70	800	550	100000	19000						
Maximun							90	1600	1200	120000	25000						

Data are reported as ng/ml, measured in serum samples after a 24 hour fast.

Table 3. *ATP7B* gene variants in ISj and Mo

ISj	exon 2	S406G	rs1801243	Heterozygous	SNP	NDV
ISj	exon 3	V456L	rs1801244	Heterozygous	SNP	NDV
ISj	intron (6)	1870-3A>G		Heterozygous		**DV**
ISj	exon 10	K832R	rs1061472	Homozygous	SNP	NDV
ISj	exon 12	R952K	rs732774	Homozygous	SNP	NDV
ISj	exon 13	A1003A	rs1801247	Heterozygous	SNP	NDV
ISj	exon 16	V1140A	rs1801249	Homozygous	SNP	NDV
ISj	intron (18)	c.3903+6C>T	rs228205	Homozygous	SNP	NDV
Mo	5'UTR	C.-75C>A	rs193922101	Homozygous	SNP	NDV
Mo	exon 2	M33T	rs184868522	Heterozygous		
Mo		S406A	rs1801243	Heterozygous	SNP	NDV
Mo	exon 3	V456L	rs1801244	Heterozygous	SNP	NDV
Mo	intron (6)	c.1870-3A>G		Heterozygous		DV
Mo	exon 10	K832R	rs1061472	Homozygous	SNP	NDV
Mo	exon 12	R952K	rs732774	Homozygous	SNP	NDV
Mo	exon 13	A1003A	rs1801247	Heterozygous	SNP	NDV
Mo	exon 16	V1140A	rs1801249	Homozygous	SNP	NDV
Mo	intron (18)	c.3669+6C>T	rs2282057	Homozygous	SNP	NDV

NVD: not disease variant; DV: disease variant; SNP: Single nucleotide polymorphism

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Conflict of Interest:

RS is Chief Scientific Officer of IGEA Pharma N.V.; she has some shares in IGEA Pharma N.V., but does not receive monetary compensation.

Ethical Approval:

All procedures were reviewed and approved by the Local Institutional Review Committee and were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent:

An informed consent was obtained from the subject participating in this research.

Additional Material:

– Metabolic Data Panel Analyses

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