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Original Article

Diminished DNA binding affinity of DMRT1 caused by heterozygous DM domain mutations is a cause of male infertility

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Abstract

The most severe form of male infertility is idiopathic non-obstructive azoospermia (NOA), a complete sperm absence in the ejaculate. We performed exome sequencing in the Croatian infertile brothers with NOA and found a variant in *DMRT1* (Doublesex and mab-3 related transcription factor 1) gene that was further assessed by the EMSA assay and molecular dynamic simulations. We additionally screened for *DMRT1* mutations in 1940 infertile men diagnosed with spermatogenic failure, 644 normozoospermic controls, and 105 females with primary ovarian insufficiency (POI) recruited to the GEnetics of Male INfertility Initiative (GEMINI) or Estonian Andrology (ESTAND) cohorts. DMRT1 p.Pro74Leu (chr9:g.842059C > T) variant was detected in infertile brothers in the highly conserved position within the DNA binding DM domain of the protein. EMSA assay showed reduced DNA binding of DMRT1^{P74L} and molecular dynamic simulations showed differences in structural and dynamical properties between the wild type protein and DMRT1^{P74L}. Plausible disease-causing *DMRT1* variants were only identified in infertile men (13/1940; 0.67%), and none in 639 fertile controls. Burden testing showed an excess of rare deleterious DM domain mutations in the infertility cohort compared to gnomAD v.4.0 population-based controls (Fisher's exact test, $p = 1.44 \times 10^{-5}$). Three rare deleterious variants in *DMRT1* were found in 104 cases of POI. The findings of this study strengthen the evidence of *DMRT1* variants being a causal factor for male infertility and provide the distribution of likely pathogenic variants across the gene. This is also the first study to suggest that *DMRT1* variants may also be linked to POI.

Keywords: genetics; infertility; exome sequencing; DMRT1

Introduction

The causes of male reproductive malfunction are highly complex and present with heterogeneous phenotypic characteristics, including anatomical anomalies, malignancies and infertility. The most severe form is non-obstructive azoospermia (NOA) characterized by spermatogenic failure, and its prevalence in male population is around 0,4%–2% [1]. Genetic defects underlying spermatogenesis failure can have significant prognostic value and lead to improvement in the clinical outcomes [2].

Next-generation sequencing, mainly whole exome sequencing (WES) is a promising tool for identifying monogenic cause of male infertility, especially in patients with NOA, which can have a

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wide spectrum of genetic variation [1]. Given the negative impact of genetic variants on reproductive success, their frequency is expected to be low in the population, which requires large cohorts to establish their relevance [3]. However, familial cases of infertility can facilitate the search for novel infertility genes and this approach was successfully applied in recent years [4].

One of the key regulators during male sex determination and testis development is the *DMRT1* (Doublesex and mab-3 related transcription factor 1) gene that encodes for a transcription factor with a zinc-finger DNA binding motif called DM domain [5]. *DMRT1* is located on the chromosomal region 9p24.3. Deletions of this region have been reported in cases presenting either 46,XY gonadal dysgenesis, 46,XY sex reversal, or 46,XY ovotesticular disorders of sex development (DSD) [6]. Deletions and missense variants in *DMRT1* were observed in patients with NOA pointing out the importance of functional characterization for each new mutation with uncertain significance [7–9].

In this study, we examined a familial case of male infertility in two Croatian brothers with NOA histologically manifesting as Sertoli cell-only syndrome (SCOS) using WES. We identified a heterozygous variant in the DM domain of the *DMRT1* gene (p.Pro74Leu; P74L; chr9:g.842059C > T) that we further examined by electrophoretic mobility shift assay (EMSA) and molecular dynamics (MD) simulations to determine its effect on the protein's affinity for DNA binding. To further clarify the contribution of rare, damaging mutations in *DMRT1* to male infertility, we screened additional WES data from the combined case–control cohorts of GEMINI (GEnetics of Male INfertility Initiative) and ESTAND (ESTonian ANDrology). The results of the study strongly suggest that heterozygous variants in the DM domain of the *DMRT1* gene are a dominant cause of male infertility, and provide the first evidence for a potential role of *DMRT1* in women with unexplained POI.

Results

Family case with two infertile brothers presenting Sertoli cell-only syndrome

Two brothers from Croatia (aged 34 and 36 years), sought medical advice and comprehensive clinical examination due to couple infertility. Brother 1 (B1) had been previously diagnosed with primary hypergonadotropic hypogonadism (HH) and presented elevated FSH and LH, and reduced testosterone (T) levels (Table 1). B1 also reported lowered sexual functions and slower growth of body hair, especially beard. Due to HH, B1 had been prescribed hormonal therapy including T replacement therapy and clomiphene citrate, however, without improvement of T values. Brother 2 (B2) had also elevated levels of FSH and LH, but T within the normal range. He had been diagnosed with testicular varicocele grade I, with a normal epididymis, and his medical history included successful treatment for urogenital infections. Both presented reduced total testicular volume (18–20 ml) compared to Croatian men [10]. Both brothers were diagnosed with idiopathic NOA. No other chronic diseases were documented.

Testicular biopsy revealed in both patients Sertoli-cell only syndrome (SCOS), tubules with a complete absence of germinal epithelium (Fig. 1). The analysis also revealed tubular fibrosis with narrowed fibrotic tubules lacking Sertoli and germ cells with different degrees of hyalinization. Both brothers had traces of tubules with thickened lamina propria called tubular shadows. Fibrotic tissue was detected in the interstitial compartment of seminiferous tubules in both brothers, although B2 had Leydig cells gathered in nodules. Traces of peritubular infiltrates of mononuclear cells were found in brothers, implying testicular inflammatory processes.

Table 1. Relevant clinical characteristics of brothers with Sertoli cell-only syndrome.

Characteristic	Brother 1 (B1)	Brother 2 (B2)
Age	34	36
Karyotype	46,XY	46,XY
AZFa, b, c microdeletions	No	No
Testicular volume left (ml)	2.9	4.2
Testicular volume right (ml)	2.6	3.5
Varicocele	No	Grade I
Cryptorchidism	No	No
Testosterone (nMol/l)	1.6	Normal
Follicle-stimulating hormone (U/l)	28.2	42.9
Luteinizing hormone (U/l)	12.8	17
Tubular inflammation	Yes	Yes

Their parents were native Croatians and familial anamnesis excluded consanguinity. The brothers had two sisters who did not report any fertility-related problems.

Identification of a genetic cause by exome sequencing

The workflow of the whole study is shown in Fig. 2. The filtering approach of the WES dataset from two brothers yielded five heterozygous variants in three genes (missense substitutions in *DMRT1*, *CTBP2*, and start loss variant in *LAMA5*). Using SpermatogenesisOnline 1.0 database [11] and literature, a heterozygous variant in the DM domain of the *DMRT1* gene (p.Pro74Leu; P74L; chr9:842059 C > T) was the most plausible variant linked to their infertility (Fig. 3A). The variant was absent from the population based databases, had not been reported before in the literature and was classified as of uncertain significance (VUS). As the predicted CADD score was high (29.90), supported by Polyphen (probably damaging) and SIFT (deleterious) scores, it was retained for further assessment. Cascade screening in the family using Sanger sequencing revealed maternal inheritance of the variant, while the father was homozygous for the reference allele (Fig. 3B and D).

In vitro DNA binding of DMRT1^{P74L} to DNA

The EMSA assay was performed to determine the impact of a heterozygous variant in the DMRT1 DM domain on protein-DNA interaction and binding ability. The analysis showed the reduced binding ability of *in vitro* translated DMRT1^{P74L} compared to wild-type DMRT1. To imitate the autosomal dominant effect of the variant in brothers *in vitro*, a heterozygous protein mixture of wild-type DMRT1 and DMRT1^{P74L} was also subjected to EMSA assay (Fig. 2C). The reduced binding ability of DMRT1 proteins with DNA was also observed suggesting that the effect of DMRT1^{P74L} has a dominant negative effect on DNA binding by WT DMRT1.

Molecular dynamic simulations of DMRT1^{P74L} DM domain

Molecular dynamics (MD) simulations provided a possible explanation of experimentally observed differences regarding DNA interactions between the wild-type DMRT1 and the DMRT1^{P74L} variant. Crystal structure, as well as the MD simulations, showed that the mutation site (residue 74) is located near the minor grove of the DNA with the sidechain orientated towards the base pairs. During MD simulations of the wild-type DMRT1, Pro74 comes

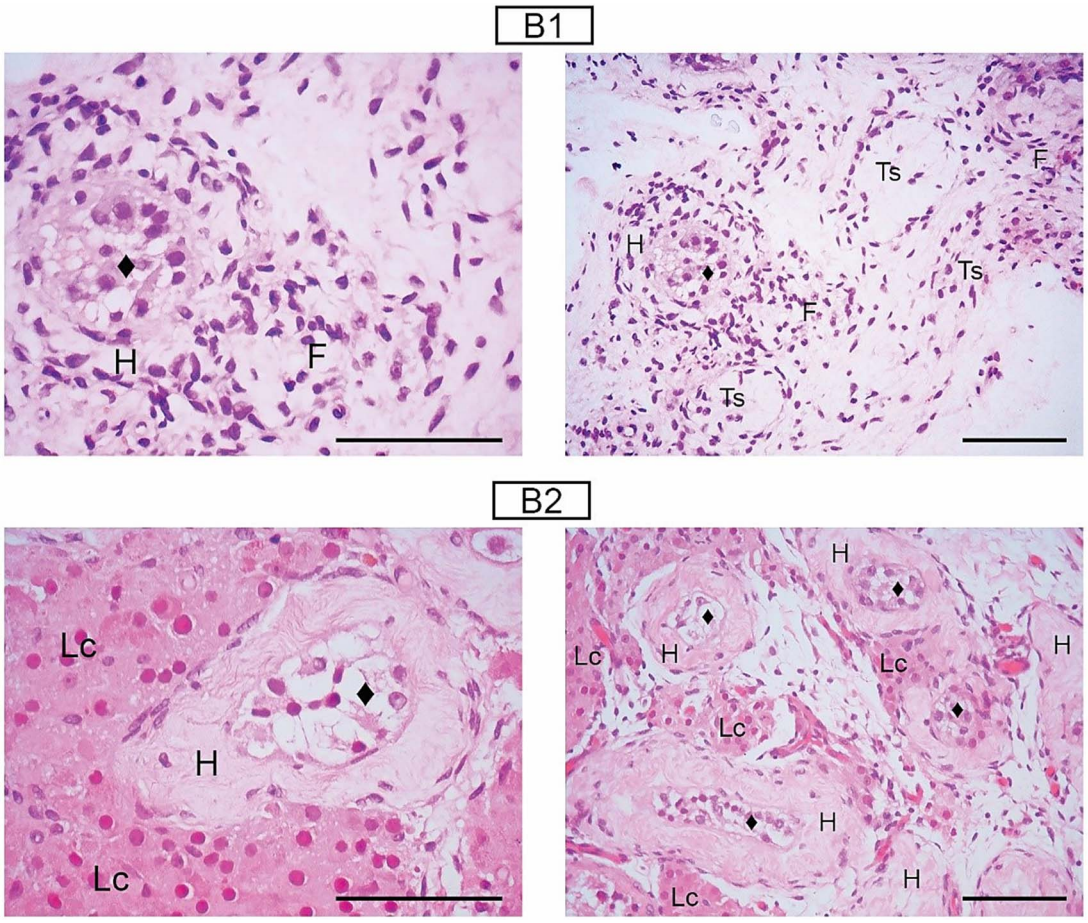

Figure 1. A) Representative images of H&E stained testicular tissue from brother 1 (B1) and brother 2 (B2). ♦—Sertoli cell-only tubules, H—Tubular hyalinization, F—Fibrosis, Ts—Tubular shadows, and Lc—Leydig cells. Scale bar shows 100 μm.

closer to the DNA minor groove compared to the initial (crystal) structure while keeping the orientation of the sidechain intact (*i.e.* orientation in the crystal structure) (Fig. 4A). On the other hand, during the MD simulations of the DMRT1^{P74L}, Leu74 moves away from the minor groove with the sidechain changing the orientation (Fig. 4B). The distance between the C_α atom of residue 74 and the base pairs of the DNA during the MD simulations (Fig. 4C), support these results. The average distances during the MD simulations are: 9.7 ± 0.6 Å for the wild-type DMRT1 and 10.8 ± 0.5 Å for the mutant DMRT1^{P74}. Further, results of cluster analysis of MD simulations (Supplementary Table 1) show that P74L mutation also affects protein flexibility and decreases the number of available protein conformations in systems without DNA. The same effect was observed in systems with and without Zn^{2+} ion bound.

Rare damaging heterozygous variants of *DMRT1* as a plausible cause of spermatogenic impairment

To explore the distribution and prevalence of rare likely pathogenic (LP) variants in the *DMRT1* gene among infertile men, 1940 idiopathic NOA or severe oligozoospermia patients recruited to the GEMINI and ESTAND cohorts were analyzed. In addition to Croatian brothers, nine infertile men were also identified as carriers of LP missense variants located in critical functional domains of DMRT1 (Table 2, Fig. 5). One VUS was identified outside of known protein domains, whereas the functional consequence of the only detected frameshift variant (p.Lys346Glufs*7; last exon) was unclear.

No LP variants were detected among normozoospermic men. The load of potentially causative variants in the combined GEMINI+ESTAND infertile compared to fertile men (13/1940 vs 0/639; 0.67% vs 0%; $P = 0.047$; Fisher's Exact Test, Supplementary Table 2) as well as population based controls from the gnomAD v.4.0 database (3221/807162, $P = 0.06$; Fisher's Exact Test, Supplementary Table 2) was not significant.

Notably LP variants showed convincing clustering pattern across the *DMRT1* gene. Six of these variants (in seven men) were mapped to the functionally most important DM DNA-binding domain [5], including the variant shared by the brothers (Fig. 5). Two mutational hotspots (Glycine 83; Valine 114) in the DM DNA binding domain were identified in five affected NOA patients. Both are evolutionarily highly conserved and have been found to be functionally important in the DNA binding domain [5, 13]. Burden testing of LP variants in the DM domain among infertile men compared to the population dataset was highly significant ($P = 1.44 \times 10^{-5}$). As burden testing across the entire gene was not significant, this analysis lends support to the functional importance of the *DMRT1* DM domain to male fertility. However, due to possible uncontrolled technical differences among gnomAD and GEMINI+ESTAND datasets, we advise caution in interpreting the absolute risk conferred by *DMRT1* DM domain mutations suggested by this burden test.

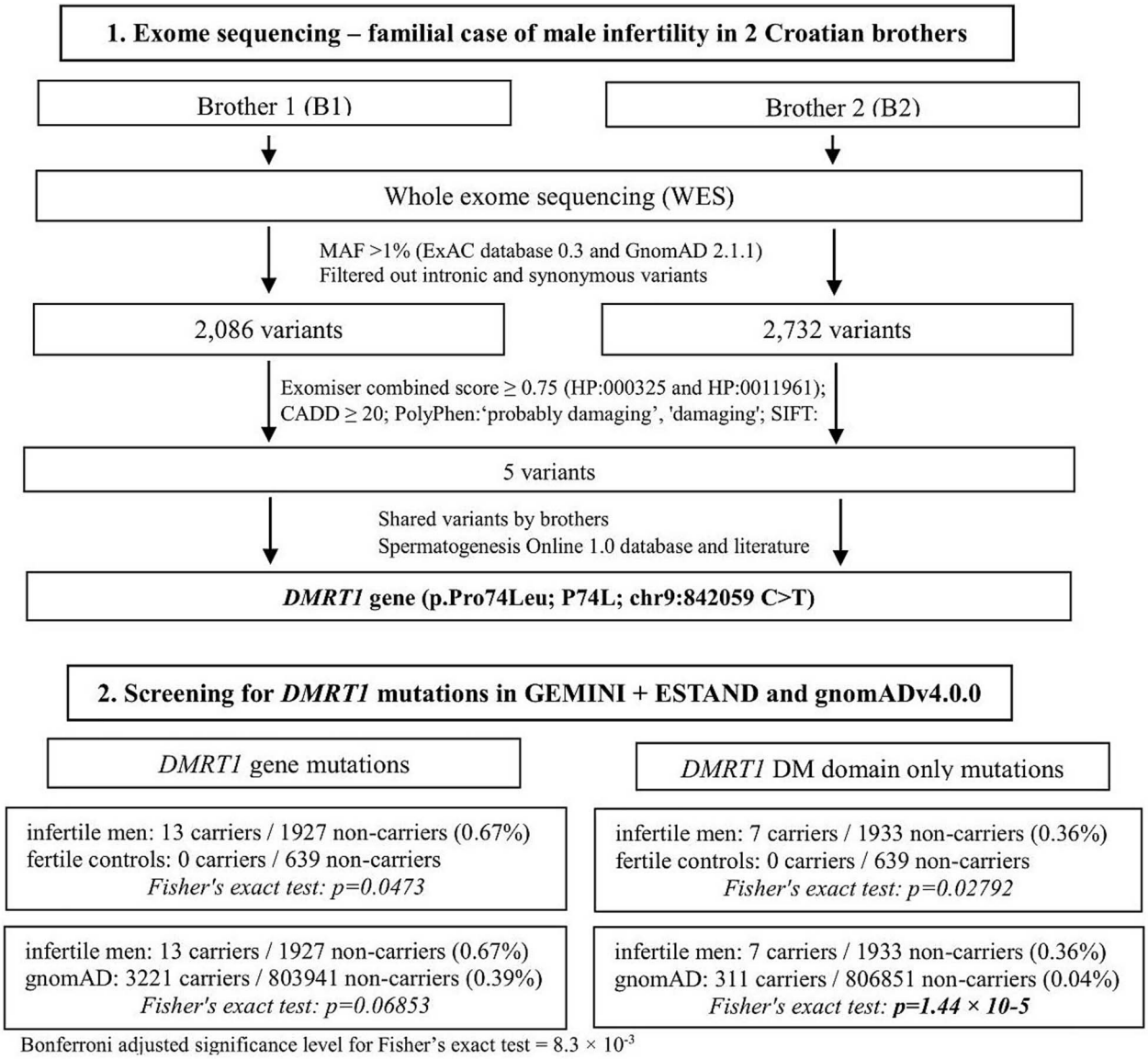

Figure 2. The workflow of the study showing the main steps of analysis.

Regional clustering of other LP variants found in the GEMINI +ESTAND patients was observed. Two missense variants were located in the double-sex mab3 related transcription factor 1 domain and three variants in the distal part encoding the Disordered II domain.

None of the patients presented other variants in their exome that were plausible causes for male infertility aside from one case with homozygous VUS in *SLC25A31* reported previously in the original GEMINI study. Both B1 and B2 were also homozygous for WFS1 p.Arg116Cys, but as neither of the brothers presented any symptoms of Wolframin syndrome linked to WFS1, this variant was excluded from further assessment. Moreover, this variant was excluded in the family case study of two brothers by the applied filtering approach due to a lower Exomiser score than the one set as a threshold (0.75).

Clinical features of *DMRT1* mutation carriers in GEMINI and ESTAND

Physical exam measurements were available for ten of the 13 male cases carrying deleterious variants in *DMRT1* (Supplementary Table 3). Eight of these cases presented with small testes (bitesticular volume 5.5–30 ml, mean = 17.8 ml), but the size distribution was not different from the overall distribution of testis volume in infertility cases assessed by GEMINI. Endocrine lab results were variable but within the ranges expected for primary gonadal dysfunction; no clear patterns were apparent regarding the genomic location of *DMRT1* mutation and endocrine levels. In addition to the two Croatian probands, three men were evaluated by testis histology, each of which was a carrier of a DM domain variant. Remarkably, all three cases presented with spermatogonial arrest, which is a rare histological finding even in the context of NOA. One of these three cases was also diagnosed with germ cell neoplasia in situ (GCNIS), verified by placental alkaline phosphatase staining (Supplementary Fig. 2). Two ESTAND patients had a medical history of cryptorchidism; both were carriers of LP variants outside of the DM domain (p.Gly132Asp, p.Ala142Val).

Rare damaging *DMRT1* variants are found in cases of primary ovarian insufficiency

Using the same filtering approach as applied to the GEMINI male infertility cohort, we identified 3 rare deleterious variants in

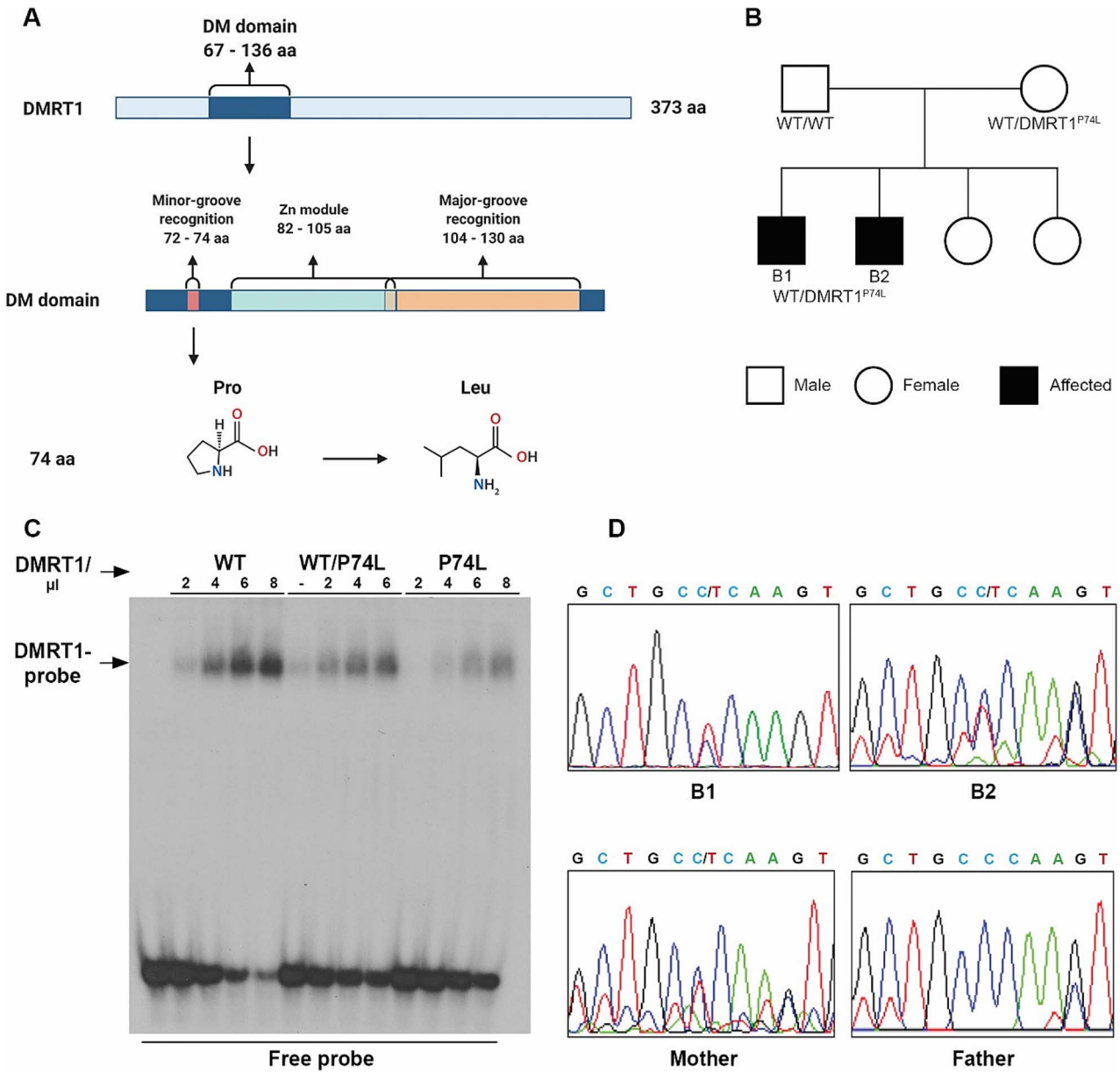

Figure 3. A) DMRT1 protein has an active DM domain that is Zn finger DNA binding motif. In the DM domain, DNA is recognized through the minor- and major-groove recognition module, while the Zn module binds Zn ions. The detected missense variant that causes a change of proline to leucine at position 74 is located in the minor-groove recognition module. Created with BioRender.com (accessed on 16 march 2022). B) Familial tree of the closest relatives of the affected brothers (black box). C) Wild-type DMRT1 and DMRT1^{P74L} subjected to electrophoretic mobility shift assay (EMSA). Per lane 2, 4, 6, or 8 μl of wild-type (WT) or mutant (P74L) protein were loaded with radiolabeled double-stranded DNA. The lanes with WT/P74L mixture contain 2 μl of the WT protein and either no mutant protein added (–) or increasing amounts of the mutant protein (2, 4, 6 μl). D) Parents of the brothers were subjected to Sanger sequencing for the *DMRT1* gene. Representative chromatograms of *DMRT1* variant P74L in brothers and mother (g.842059C > T) and wild-type in father (g.842059C).

DMRT1 in 104 cases of primary ovarian insufficiency (Table 2, Fig. 5, Supplementary Table 3). Notably, the variant DMRT1 p.Gly83Val was shared between a POI and a NOA case and the other two variants DMRT1 p.Cys79Phe and p.Ser329Ile clustered closely with the findings in infertile men.

Discussion

Mutations in *DMRT1* have been linked to a wide variety of human infertility phenotypes, ranging from complete XY gonadal dysgenesis to isolated male infertility. This phenotypic variability suggests that human genetic variation changes DMRT1 function on a quantitative scale, and, perhaps, functional effects of *DMRT1* variants are influenced by genetic background [14] and environmental factors.

We identified a novel heterozygous *DMRT1* variant in Croatian brothers diagnosed with NOA and SCOS affecting the DNA binding DM domain (p.Pro74Leu; chr9:g.842059C > T). DMRT1^{P74L} alone, or in a heterozygous mixture with wild-type DMRT1 showed a reduced binding to DNA compared to wild-type. This was further supported by MD simulations that showed reduced affinity of mutated protein for the DNA minor groove binding. The observed difference can be explained by the steric effect of the larger branched isobutyl group of the Leu side chain compared to the

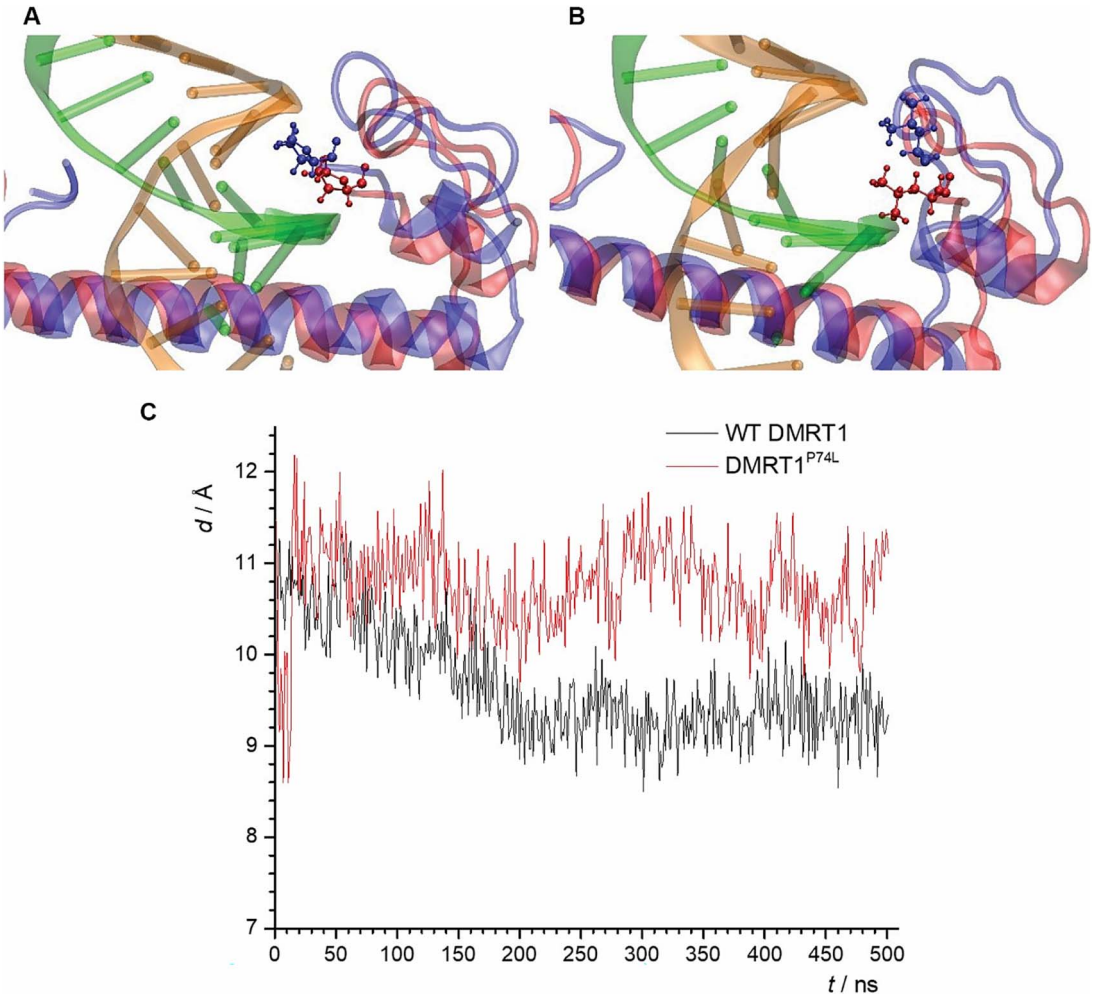

Figure 4. A) Position of Pro74 of wild-type DMRT1 B) and Leu74 of DMRT1^{P74L} relative to the minor groove of the DNA at the beginning of the simulation (red) and the end of the simulation (blue). Amino acid 74 (mutated site) is shown as a ball-and-stick representation. C) Distance between the C_α atom of the residue 74 and the base pairs of the DNA for the wild-type DMRT1 (black) and the DMRT1^{P74L} (red) protein.

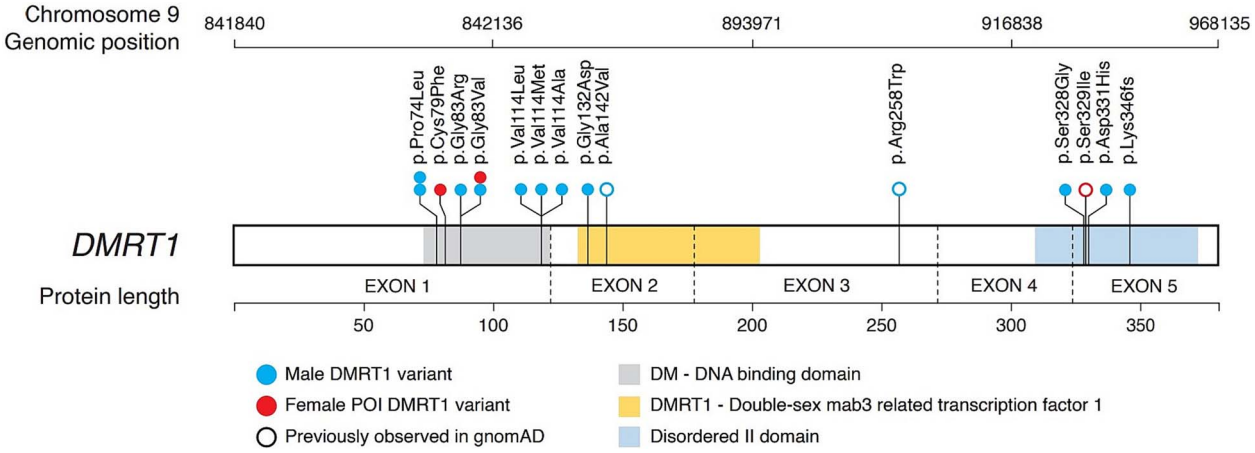

Figure 5. Deleterious DMRT1 mutations found in cases of infertility cluster at protein domains and codons. The locations of the 14 variants reported in this study are projected onto a model of the protein. Variants are colored by sex of the case(s) carrying the variant. Open circles indicate variants observed in gnomAD, while closed circles indicate novel variants.

Pro side chain which consists of three ring-forming atoms, higher hydrophobicity of Leu compared to Pro [15] and removal of steric restriction in the DMRT1^{P74L}. Furthermore, cluster analysis of trajectories showed that the DMRT1^{P74L} is more rigid than the wild-type DMRT1 protein which might affect the availability of the DNA binding conformations of the protein in solution and can contribute to the weaker DNA binding of the DMRT1^{P74L} compared to the wild type.

Previously, DM domain missense variants were found in the Zinc finger motif (p.Arg80Ser; p.Tyr84Cys) [16, 17] or the

Table 2. DMRT1 variant and clinical information from the GEMINI and ESTAND cohorts.

GEMINI/ESTAND ID	Phenotype/clinical info	DMRT1 Protein Domain	Genomic Position	cDNA	AAChange	ACMG Classification	Sanger Verified
ZAGREB-B1	NOA, mixed atrophy; extremely low bilateral volume (6.5)	DM DNA-binding domain	chr9:842059_C > T	c.221C > T	p.Pro74Leu	LP	Yes – Zagreb
ZAGREB-B2	NOA, tubular fibrosis, SCOS; extremely low bilateral volume (7.7 ml)	DM DNA-binding domain	chr9:842059_C > T	c.221C > T	p.Pro74Leu	LP	Yes – Zagreb
GEMINI_POI-1	POI, diagnosed at age 35	DM DNA-binding domain	chr9:842074_G > T	c.236G > T	p.Cys79Phe	LP	No DNA
GEMINI-743	NOA	DM DNA-binding domain	chr9:842085_G > C	c.247G > C	p.Gly83Arg	LP	Yes
GEMINI_POI-2	POI, diagnosed at age 32	DM DNA-binding domain	chr9:842086_G > T	c.248G > T	p.Gly83Val	LP	No DNA
GEMINI-899	NOA, spermatogonial arrest with occasional spermatids +30%SCOS +20% sclerohyalinosis	DM DNA-binding domain	chr9:842086_G > T	c.248G > T	p.Gly83Val	LP	Yes
GEMINI-71	NOA, extremely Low bilat vol (12 ml)	DM DNA-binding domain	chr9:842178_G > C	c.340G > C	p.Val114Leu	LP	Yes
GEMINI-337	spermatogonial arrest, GCNIS (Testicular germ cell neoplasia in situ); few spermatids	DM DNA-binding domain	chr9:842178_G > A	c.340G > A	p.Val114Met	LP	Yes
GEMINI-1061	NOA	DM DNA-binding domain	chr9:842179_T > C	c.341 T > C	p.Val114Ala	LP	Yes
ESTAND2*	Severe oligo, low bilateral volume (0 + 18), unilateral cryptorchidism	Doublesex- and mab-3-related transcription factor 1-like	chr9:847000 G > A	c.395G > A	p.Gly132Asp	LP	Yes - Estonia
ESTAND3*	Severe oligo, low bilateral volume (29 ml)	Doublesex- and mab-3-related transcription factor 1-like	chr9:847030 C > T	c.425C > T	p.Ala142Val	LP	Yes - Estonia
GEMINI-653	NOA		chr9:894145_C > T	c.772C > T	p.Arg258Trp	VUS	Yes
ESTAND1*	NOA, low bilat volume (23 ml), bilateral cryptorchidism	Disordered II	chr9:967999 A > G	c.982A > G	p.Ser328Gly	LP	Yes –Estonia
GEMINI_POI-3	POI, menses stopped at age 26, family history of POI	Disordered II	chr9:968003_G > T	c.986G > T	p.Ser329Ile	LP	No DNA
GEMINI-1008	NOA	Disordered II	chr9:968008_G > C	c.991G > C	p.Asp331His	LP	Low DNA quality – poor sanger trace 3x
GEMINI-944	NOA, Mixed atrophy with incomplete spermatogonial arrest in the right testis and spermatid arrest in the left testis.	Disordered II	chr9:968053_AA>-	c.1036_1037del	p.Lys346Efs*7	VUS - LP	Yes

ACMG, American College of Medical Genetics and Genomics; LP, likely pathogenic; VUS, variant of unknown significance. [a]Cases ESTAND1–3 were originally published by [12].

major-groove recognition helix (p.Arg111Gly; p.Arg111Met) [5] in complete gonadal dysgenesis or sex reversal. The heterozygous variant p.Leu139Gln, located outside the DM domain, was also proposed as pathogenic in complete gonadal dysgenesis [18]. In NOA patients with maturation arrest, SCOS, and cryptozoospermia heterozygous missense variants (p.Asn224Ser, p.Asp331His) were outside the DM domain, in the less conserved regions with undetermined functional effects [19, 20]. A recent study by Emich

et al. showed a pronounced functional effect for one DM domain variant (p.Met115Lys) associated with isolated male infertility by luciferase assay [7].

We further compared a cohort of 1940 men with spermatogenic impairment to a large sample set of population controls and identified a significant burden of rare, damaging mutations in *DMRT1*, which was significantly enriched in DM domain variants. *DMRT1* mutation carriers showed several significant clinical similarities: testis biopsy showed spermatogonial arrest in three cases; two cases reported a history of cryptorchidism, and 1 case presented with GCNIS. The Croatian brothers' testis showed even more damaging phenotype, exhibiting as SCOS, tubular fibrosis, and reduced Leydig cells, while B1 had also HH diagnosed.

Elegant mouse experiments have shown that DMRT1 has multiple functions in the gonad throughout the mammalian lifecycle. Mice deficient in *DMRT1* have severe defects in testis development, including defects in Sertoli cell differentiation [21]; presumably, this requirement of *DMRT1* for testis development contributes to the small testis size and cryptorchidism seen in *DMRT1* mutation carriers. Additionally, DMRT1 plays a critical role in male germ cells' maturation, balancing the fate of spermatogonia between mitotic and meiotic programs by repressing expression of *STRA8* and other genes induced by retinoic acid [22]. In that respect, *DMRT1* is a gene that exemplifies the testicular dysgenesis syndrome which suggests that, testicular germ cell cancer, poor semen quality, cryptorchidism and some forms of hypospadias all are due to a dysgenic fetal development of the male gonad [23].

Integrating the genomic location of *DMRT1* changes with the patient clinical data, we could organize the gene into 4 regions: the previously mentioned DM domain, the DMRT1 domain, an unannotated linker region, and a Disordered II domain (Fig. 5, Table 2). The 9 case variants in the DM domain (including the brothers) have the strongest hallmarks of disease mutations: none of these are reported in gnomAD, they cluster into 4 amino residues, and they have on average the highest CADD scores of the variants detected in cases. All cases linked to these mutations have NOA or POI. The two patients with variants in the DMRT1 domain were diagnosed with a milder condition, severe oligozoospermia, and these variants were previously observed in population sequencing. The variants in the linker and the Disordered II regions are all linked to NOA or POI cases and contain a mixture of population frequencies (observed and not observed in gnomAD). Clearly the genotype–phenotype mapping of variants in the gene will be complex and will benefit from *in vitro* and *in vivo* assays if possible. Very importantly, all of the variants observed in the study were seen as heterozygotes. While the original reports of mice with heterozygous null alleles appeared to have completely normal reproductive parameters, more recent work has shown clearly that missense changes in the DM domain can be a dominant cause of mouse male infertility [24].

The role of *DMRT1* mutations in primary ovarian insufficiency (POI) in humans has not yet been established. Interestingly, opposite to male germ cells, *DMRT1* appears to activate *Stra8* expression in female fetal germ cells in mice [6]. Mice that lack *DMRT1* in the fetal ovary have a decreased number of primordial follicles in the juvenile ovary with a reduced activation of *Stra8* [25]. A reduced ovarian reserve at birth is consistent with the POI phenotype observed in human female carriers of deleterious *DMRT1* variants. One of the POI cases shares the same amino acid substitution with two NOA cases in the DNA binding domain (p.Gly83Val).

In this study we were able to assess the frequency of rare damaging *DMRT1* variants across two independent male infertility cohorts (GEMINI+ESTAND). *DMRT1* deletions have also been reported in two prior studies of CNVs in NOA cohorts. These early GEMINI studies found *DMRT1* deletions in 2/323 (0.6%) men of largely European ancestry [9], and 3/970 (0.3%) of NOA Han Chinese [26]. Combined with the two cohorts reported here, we observe a consistent frequency of around 0.7% with heterozygous *DMRT1* mutations across a wide range of ethnicities. When CNVs and SNV/indel data are combined on the same cohorts this number may approach 1%. The frequency of rare *DMRT1* variants in the POI cohort is even higher (3/104, 2.8%) but the sample size is small.

In conclusion, our findings strongly suggest that the diminished DNA binding affinity of DMRT1 resulting from rare, dominant mutations within the DM domain could be a significant causal factor for male infertility. Our findings provide further evidence supporting the relevance of *DMRT1* in the context of male infertility and potentially in relation to POI.

Materials and methods

Ethics statement

The study was approved by the Ethics Committee of the University Hospital Centre, Zagreb, Croatia with the reference number 02/21 JG. The informed consent was obtained from the infertile brothers and their parents for use of their clinical data solely for scientific purposes. The ESTAND study was approved by the Ethics Review Committee of Human Research of the University of Tartu, Estonia (permission no. 74/54 and 118/69 with last amendment 288/M-13). GEMINI was approved was approved by the Ethics Committee of all collaborative centers: IRB protocols #201107177 and #201109261 for Washington University in St. Louis, USA, #012049 for University of Utah, and #20243 for Oregon Health & Sciences University [1]. The procedures were carried out according to the regulations of the Declaration of Helsinki.

Clinical examination of cases

Familial case of male infertility

Two infertile brothers from Croatia were referred to the University Hospital Zagreb, Croatia for the evaluation of male infertility. A routine andrological pipeline included examination by an andrologist, collection of medical history, reproductive hormone levels, and testicular ultrasound according to European Association of Urology (EAU) guidelines [27] (Table 1). The examination of the brothers excluded the following known causes of male infertility: obstruction of the seminal ducts, testicular anomalies such as cryptorchidism, hypospadias, testicular traumas, and radio- or chemotherapy. The brothers further underwent routine screening for *AZF* microdeletions and karyotyping. Accordingly, the brothers were referred for testicular biopsy by testicular sperm extraction (TESE). Family history data, as obtained by the brothers, did not point to any other infertility cases in the family. Follow up clinical and phenotype information for their mother was not available to assess for potential POI in this carrier of a *DMRT1* variant.

Histological phenotyping of testicular tissue

Testicular tissue of the brothers was collected by TESE at University Hospital Centre, Zagreb, Croatia. Multiple tissue pieces were taken bilaterally and divided into half either for histopathological analysis or for further cryopreservation. Immediately after the biopsy, tissue was fixed in Bouin's fluid and tissue sections were stained with hematoxylin and eosin. Histopathological analysis of the tissue was performed by an experienced andrologist.

GEMINI and ESTAND

Physical exam measurements were available for ten additional cases in the combined infertility cohort. In the GEnetics of Male INfertility Initiative (GEMINI), men were confirmed to have azoospermia or severe oligozoospermia according to the AUA/ASRM guidelines [28, 29] and based on physical examination (testis volume), endocrine measures (FSH, LH, and T) and histological findings if available, as previously described [1]. The andrological phenotyping of Estonian Andrology (ESTAND) cohort has been described in detail by [30]. In both cohorts, whole exome sequencing (WES) was performed only for idiopathic cases, excluding all known genetic (Y-chromosomal microdeletions, chromosomal abnormalities, CFTR mutations) and non-genetic causes of male infertility.

Whole exome sequencing

A familal case of male infertility in two brothers

Total genomic DNA was isolated from the peripheral blood samples of Brother 1 (B1) and Brother 2 (B2) using the standard extraction procedure (Invitrogen™ iPrep™ PureLink™ gDNA Blood Kit). Whole exome sequencing (WES) was performed in Macrogene Inc using the NovaSeq6000 platform, Agilent SureSelect XT_V5 + UTR library preparation kit.

Obtained sequences were aligned to the human reference genome version hg38 [31] using the BWA-MEM software [32]. Variants were called with the HaplotypeCaller algorithm from GATK [33] and annotated with Jannovar [34] using the transcript definition database from UCSC Genome Browser.

GEMINI and ESTAND

In the GEMINI cohort, WES was performed on 2251 individuals including 1503 men diagnosed with spermatogenic failure (most with idiopathic NOA), 104 females with primary ovarian insufficiency (POI), and 644 controls (639 male and 5 female), as described in [1] (NIH dbGaP accession number phs003115.v1.p1). Additionally, the WES dataset of 437 NOA or severe oligozoospermia cases recruited to the ESTAND cohort [30] was utilized for targeted lookup. For these ESTAND participants the WES data generation was performed at the NGS Sequencing core laboratory of the Institute for Molecular Medicine Finland (FIMM) Technology Centre, Helsinki, Finland. The details are provided elsewhere [12]).

WES data for 104 cases of primary ovarian insufficiency (POI) were generated at Washington University School of Medicine and are available through the NIH dbGaP database as accession phs001174.v1.p1.

Selection of relevant genetic variants in the brothers

All variants with population minor allele frequency (MAF) higher than 1% in ExAC database 0.3 and GnomAD database release 2.1.1 [35] were removed from both samples' data. All intronic and synonymous variants were filtered out resulting in 2086 variants in the B1 and 2732 variants in the B2. Filtered data were then assessed by Exomiser software [36] using the search terms male infertility (HP:000325) and non-obstructive azoospermia (HP:0011961). Further investigation was focused on variants shared by B1 and B2 and presenting Exomiser combined score ≥ 0.75, Combined Annotation-Dependent Depletion (CADD) ≥ 20, PolyPhen and SIFT prediction at least 'probably damaging', and 'deleterious', respectively. The functions of the retained genes in reproductive system were assessed using SpermatogenesisOnline 1.0 database [11] and literature.

Analysis of *DMRT1* variants in the GEMINI and ESTAND cohorts

Population Sampling Probability (PSAP) software was used to prioritize likely causative mutations from the WES data of the GEMINI cohort ([1], [37]). PSAP prioritized variants in the GEMINI cohort were further filtered by retaining only heterozygous *DMRT1* variants with CADD16phred scores greater than 20 and PSAP p-value less than 1×10^{-3}. The GEMINI cohort included the Croatian brothers, who were sequenced independently (i.e. a second time) and without knowledge of the *DMRT1* mutation.

DMRT1 variants in the ESTAND cohort were identified from the generated WES dataset during a hypothesis-based study targeting likely pathogenic (LP) and pathogenic (P) variants in 660 candidate genes for male infertility [12]. Pathogenicity of retained variants in both cohorts was assessed following the American College of Medical Genetics and Genomics (ACMG) guidelines [38].

Gene-based burden testing was performed comparing the load of DMRT1 disease-causing variants in the combined GEMINI+ESTAND male patient dataset ($n = 1940$ infertility cases) with either GEMINI+ESTAND controls ($n = 639$ normozoospermic subjects) or the dataset of population-based controls with unknown fertility status retrieved from gnomAD v4.0 database ($n = 807\,162$). The sum of allele counts for each variant meeting equivalent filter standards to the GEMINI cohort (CADD16Phred score > 20, maximum population MAF < 0.01) was used for burden testing with gnomAD controls. As we were unable to identify and/or control for individuals in gnomAD that carry multiple *DMRT1* variants, our estimates of control carrier frequency may be artificially high. Genetic variants were annotated with the reference to ENSEMBL transcript ENST00000382276.8. Fisher Exact Test was used to test for statistically significant differences in variant proportions and Bonferroni correction was used to determine the adjusted significance level of 8.3×10^{-3}.

Validation of *DMRT1* variants by Sanger sequencing

Variants in the *DMRT1* gene found in the affected brothers as well as in the combined infertility cohort were confirmed by Sanger sequencing (Supplementary Fig. 1). DNA samples from the three POI cases and sample GEMINI-1008 were not available for Sanger sequencing. To investigate the inheritance of the prioritized *DMRT1* variant in the brothers from Croatia, genomic DNA was extracted from saliva samples of both parents and subjected to Sanger sequencing.

In vitro DNA binding

EMSA was performed as previously described [39] except that substrates were end labeled with T4 polynucleotide kinase (NEB). Mutations were incorporated into hDMRT1 by overlap-extension PCR [40] with a T7-hDMRT1 (pDZ142) plasmid clone as a template. The mutated products were subcloned back into pDZ142. After confirming the sequence by Sanger sequencing, proteins were translated *in vitro* with the TNT Quick Coupled transcription/-translation system (Promega). The expression level of *in vitro* translated proteins was evaluated by Western blot analysis using 10 μl, 5 μl, and 2.5 μl of the same prep of proteins used for the EMSA analysis demonstrating both proteins were produced at similar levels.

Molecular dynamic simulations

Starting from the available crystal structure of human DMRT1 protein in complex with DNA fragment (pdb code: 4yj0) [5], the following systems were generated *in silico*: (i) DMRT1 protein alone, (ii) DMRT1 protein in complex with zinc ion (Zn^{2+}), and (iii) DMRT1 protein in complex with Zn^{2+} and DNA. Each system was prepared as the wild-type (WT) DMRT1 protein and as the $DMRT1^{P74L}$ which resulted in six systems in total. Systems were prepared for molecular dynamics (MD) simulations, details are given in SI. After energy minimization and equilibration (details in SI), systems were subjected to 500 ns of production phase MD simulations. Each of the six systems was subjected to three independent MD simulations. Periodic boundary conditions (PBC) were applied and TIP3P model of water molecules [41] was used. Analyses of MD simulations were performed using VMD [42] and GROMACS analyzing tools [43]. Details of the analyses are provided in Supplementary Methods.

Supplementary data

Supplementary data is available at *HMG Journal* online.

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Conflict of interest statement: Authors MLK, LŽ and MB are/were employed full or part time by the company Genom. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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