INTRODUCTION

Hypohidrotic ectodermal dysplasia (HED) is a rare congenital disorder characterized by abnormal development of ectodermal-derived structures, resulting in defects in skin appendages (Chassaing et al., 2006). Sparse hair (hypotrichosis), abnormal or missing teeth (anodontia or hypodontia), and reduced ability of eccrine sweat glands (hypohidrosis) are the main signs of the disorder (Cluzeau et al., 2011). Additional dysmorphic features may be associated with HED, including frontal bossing, an everted nose, prominent thick lips, a pointed chin, rings under the eyes, and the sporadic absence of nipples (Cluzeau et al., 2011; Naqvi et al., 2011).

BACKGROUND

Hypohidrotic ectodermal dysplasia (HED) is the most common form of ectodermal dysplasia and is mainly associated with mutations in the EDA, EDAR, and EDARADD responsible for the development of ectodermal-derived structures. HED displays different modes of inheritance according to the gene that is involved, with X-linked EDA-related HED being the most frequent form of the disease.

METHODS

Two families with tooth agenesis and manifestations of HED underwent clinical examination and EDA, EDAR, and EDARADD genetic analysis. The impact of the novel variant on the protein was evaluated through bioinformatics tools, whereas molecular modeling was used to predict the effect on the protein structure.

RESULTS

A novel missense variant was identified in the EDAR (c.287T>C, p.Phe96Ser) of a female child proband and her mother, accounting for autosomal dominant HED. The genetic variant c.866G>A (p.Arg289His) in EDA, which has been previously described, was observed in the male proband of another family confirming its role in X-linked HED. The inheritance model of the missense mutation showed a different relationship with X-linked HED and non-syndromic tooth agenesis.

CONCLUSION

Our findings provide evidence of variable expression of HED in heterozygous females, which should be considered for genetic counseling, and different modes of inheritance related to tooth development.
HED depends on mutations in one of the several genes encoding elements of the ectodysplasin A (EDA) signaling pathway, which plays an important role in embryonic ectodermal development (Cluzeau et al., 2011).

The EDA gene encodes for different splice variants and the most common isofrom is EDA-A1, a trimeric type II transmembrane protein characterized by a short collagen segment and a tumor necrosis factor (TNF) domain in its extracellular region. The proteolytic cleavage at the furin site near the collagen domain releases the EDA ligand into the extracellular space as a soluble protein (Elomaa et al., 2001). The trimeric active form interacts with the EDA receptor (EDAR) by its C-terminal TNF homologous domain. The binding of EDA-A1 to EDAR results in the enrollment of the EDAR-associated death domain (EDARADD). EDARADD interacts with EDAR by the death domain (DD) of both proteins and mediates the activation of an intracellular NF-κB signal-transducing pathway, essential for the initiation, formation, and differentiation of skin appendages (Kumar et al., 2001; Mikkola & Thesleff, 2003; Reyes-Reali et al., 2018).

Mutations in EDA, EDAR, and EDARADD account for almost 90% of HED cases (Cluzeau et al., 2011). The most common HED is the X-linked form (MIM 305100) resulting from mutations of EDA (MIM 300451) located on chromosome Xq12-q13.1. It is rare that mutations in the same gene underlie an isolated non-syndromic hypodontia that is not associated with anomalies in other ectodermal appendages or organs (Ayub et al., 2010). Mutations in the gene encoding EDAR (MIM 604095), located on chromosome 2q11-q13, or EDARADD (MIM 606603), found on chromosome 1q42-q43, cause autosomal recessive (MIM, 224900 and 614941, respectively) and dominant (MIM, 129490 and 614941, respectively) HED forms (Headon et al., 2001; Monreal et al., 1999), clinically indistinguishable from the X-linked EDA form (Cluzeau et al., 2011).

In this study, two children with clinical manifestations of HED and their parents were investigated. Two variants in EDA and EDAR were identified and analyzed to determine their clinical significance and inheritance mode.

### 2 | MATERIALS AND METHODS

#### 2.1 | Ethical compliance

Written informed consent for retrospective data collection, molecular studies, and manuscript submission for review and possible publication was obtained from the children’s parents, and the study was conducted in accordance with the principles of the Declaration of Helsinki. The study did not require approval from the ethics committee system.

#### 2.2 | Patients

Two young patients who had been referred to the Interdepartmental Coordination Centre for Rare Diseases of the University Hospital – The United Hospitals (Ospedali Riuniti) of Ancona (Italy) for diagnostic purposes were investigated. The personal and family histories of the patients were recorded and they were subjected to a physical examination. Blood samples were collected from the probands and their parents for molecular genetic testing.

#### 2.3 | Variant detection

Genomic DNA was extracted from peripheral blood using standard laboratory techniques. All coding exons and their flanking regions of EDA, EDAR, and EDARADD were amplified by PCR using specific primers and DreamTaq Hot Start DNA polymerase (Thermo Scientific). Results of PCR amplification were visualized on agarose gel and subsequently PCR fragments were purified with ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems) or, if necessary, with the MinElute Gel extraction kit (Qiagen). Direct sequencing was performed using the BigDye Terminator v.1.1 Cycle Sequencing kit according to the manufacturer’s instructions and analyzed using the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Forward and reverse sequences were aligned to references using Unipro UGENE software (Okonechnikov et al., 2012).

Sequence variations were indicated according to mutation nomenclature, with +1 corresponding to adenine of the ATG translation initiation codon of the reference sequences NM_001399.5 (EDA), NM_022336.4 (EDAR), and NM_145861.4 (EDARADD). The detected EDA and EDAR variants were submitted to the ClinVar database with the accession numbers ClinVar:SCV001366090 and ClinVar:SCV001366089, respectively.

The population genetics data from 1000 Genomes (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/), Single Nucleotide Polymorphism Database (dbSNP, https://www.ncbi.nlm.nih.gov/snp), and Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/) were used to rule out population polymorphisms.

A sequence variant was considered as disease causing when the following three conditions were met: (a) it co-segregated with the HED phenotype in families, (b) the substitution involved a conserved amino acid between distant species, and (c) the sequence variant was absent in a panel of 100 chromosomes from unaffected unrelated individuals. To assess the last condition, restriction enzyme digestions with BbvI and MboII and analysis of the restriction patterns were
used to screen 100 DNA samples for the absence of variants in \textit{EDA} and \textit{EDAR}, respectively.

### 2.4 | Bioinformatics analysis

In silico prediction of the impact of the novel missense variant on the protein product was carried out using Sorting Intolerant from Tolerant (SIFT) (Vaser et al., 2015), Rare Exome Variant Ensemble Learner (REVEL) (Ioannidis et al., 2016), NetDiseaseSNP 1.0 server (Johansen et al., 2013), Mutation Taster (Schwarz et al., 2014), and MutPred2 tool (Pejaver et al., 2017). Multiple sequence alignments of EDA and EDAR proteins with their homologues were performed using the Constraint-based Multiple Alignment tool (COBALT) (Papadopoulos & Agarwala, 2007).

### 2.5 | Molecular modeling

Structural modeling of the wild-type and mutant EDAR protein was obtained with SWISS-MODEL (Waterhouse et al., 2018). The models were visualized using Chimera 1.14 (http://www.rbvi.ucsf.edu/chimera/) (Pettersen et al., 2004). The reliability of the structure was assessed through Molprobity and QMEAN. Finally, the DUET server (http://biosig.unimelb.edu.au/duet/stability) was used to evaluate protein stability (Pires et al., 2014). In order to obtain a general view of the predicted effect of the new variant, the protein–protein docking of EDAR/EDA was determined using ClusPro 2.0 (https://cluspro.bu.edu/; Kozakov et al., 2017). The crystal structure of the EDA protein (PDB ID: 1rj7) was considered for the docking prediction.

### 3 | RESULTS

#### 3.1 | Clinical findings

The proband in family A was a 30-month-old female born to non-consanguineous parents at term by induced childbirth due to oligoamnios. She exhibited her first tooth at 14 months, regular bowel and nail dysplasia of the big toes at the base. In addition, she displayed conical mandibular teeth and the absence of lateral incisors, brittle hair, a lower right auricle, frontal bossing, and eutrophic skin. Her father suffered from...
Crohn’s disease; her mother showed sparse hair, the absence of lateral incisors, and unerupted permanent canines, while her older brother displayed agenesis of the upper lateral left incisor. There was a family history of tooth agenesis (lateral maxillary incisors), early baldness (grandfather and maternal uncle), and fragile nails with normal sweating (Figure 1).

The proband in family B was a 28-month-old male, born at term after a normal pregnancy by cesarean section due to a breech position. At 17 months, he was hospitalized for minimum pericardial effusion detected during a routine examination. He exhibited a phenotype with very light colors, conical teeth, mild bilateral dysplasia of the first toe, a tendency for ocular convergence, retractile testicles, and the absence of both maxillary and mandibular deciduous lateral incisors. In addition, the skin of his lower limbs appeared slightly dry and his hair was thin. His weight (50° pct), height (50° pct), growth curves, and psychomotor development were normal. His mother was missing her maxillary lateral incisors; the lack of dental eruption and the diagnosis of HED were reported in maternal lineages (Figure 2).

### 3.2 Genetic findings

The sequencing of EDAR and EDARADD was performed in the proband of family A because no variants were found in EDA. Screening of the entire coding sequences and the intron–exon junctions revealed a novel missense variant NM_022336.4:c.287T>C (p.Phe96Ser) within EDAR (Figure 1) in heterozygote form (ClinVar:SCV001366089). The mother of the proband carried the variant (heterozygous), whereas the father was wild-type at c.287. This variant occurred at the highly conserved amino acid residue pPhe (Figure 3a) changed to serine, and it has been not reported in Clinical Variant, dbSNP databases, or gnomAD database.

SIFT (Vaser et al., 2015) and REVEL (Ioannidis et al., 2016) predicted the variant to be “deleterious” and “likely disease causing”, respectively. Furthermore, the predictions by NetDiseaseSNP 1.0 server (Johansen et al., 2013) and Mutation Taster (Schwarz et al., 2014) foretold the single non-synonymous SNP as disease causing. Mutpred2 (Pejaver et al., 2017) pointed to the pathogenicity of the variant and a molecular mechanism of the amino acid substitution with a gain of disulfide linkage at C93, −3 residues close to the substitution.

In family B, sequencing of the eight coding exons and their intron flanking regions of EDA revealed the missense variant c.866G>A (Figure 2), resulting in the substitution of arginine with histidine at position 289 (p.Arg289His) (NM_001399.5) of the EDA protein (Figure 3b). It was reported in all of the databases considered (dbSNP:rs876657641, ClinVar:VCV000228257.2) with the exception of gnomAD.

**Figure 2** Pedigree of family B carrying a mutation in the EDA gene. The c.866G>A (p.Arg289His) variant was identified both in the EDA gene of the proband (hemizygote) and his mother (heterozygote). Each of the main clinical features of HED (oligodontia, hypotrichosis, and hypohidrosis) was indicated individually, whereas sporadic and mild signs were included in “other symptoms of HED”. The black arrow points to the proband.
This variant was present in both the male proband (hemizygote) and his mother in heterozygous form, but it was not found in his father. Nucleotide sequence analysis of 100 unaffected unrelated subjects showed that both of the variants do not represent polymorphic sites.

3.3 | Protein structure and stability prediction

The protein structure model was generated using the SWISS-MODEL server considering the TNF receptor superfamily member 16 (PDB ID: 3buk1.c; Sequence identity: 29.29%) as a template. The reliability of the models was assessed through Molprobity web service and QMEAN. The scores obtained for EDAR wild-type were: Molprobity score 1.95, Ramachandran Favored 92.04%, Ramachandran Outliers 0.88%, Rotamer Outliers 1.03%, and QMEAN −3.46. On the other hand, the estimates obtained for the mutated EDAR were Molprobity score 0.99, Ramachandran Favored 92.04%, Ramachandran Outliers 0.88%, Rotamer Outliers 0.00%, and QMEAN −3.82. The protein structure stability was calculated using DUET, which predicted Phe96Ser in EDAR as destabilizing with negative free energy change value ($\Delta\Delta G$: −1.247 kcal/mol).

The analysis of the two protein structures revealed different interatomic distances as shown in Figure 4. The binding between EDAR and its EDA ligand was confirmed through the docking analysis, but it disappeared as a result of the docking with the mutated EDAR protein.

DISCUSSION

The congenital absence of one or more permanent teeth can be non-syndromic, with patients only showing tooth anomalies or syndromic, with the tooth defect as just one of the symptoms of a more complex syndrome. Both cases can be caused by mutations in the same genes, usually $EDA$, $EDAR$, and $EDARADD$, which are involved in the same signal pathway essential for ectodermal structure development (Zeng et al., 2017). In particular, X-linked HED and EDA-related non-syndromic tooth agenesis could be considered the same disease with different expressivity (Zeng et al., 2017).

The missense variant p.Arg289His in $EDA$ was identified in family B in which dental anomalies are the main symptoms in the proband, his mother, grandfather, uncles, and cousins (Figure 2). In addition to tooth agenesis and defects in the
shape of his teeth, the child displayed further signs, namely xerotic skin and a phenotype with very light colors. The latter feature was also common in his cousins (III:15 and IV:1) as children. Sparse hair and eight missing teeth with a lack of bone were the clinical manifestations of the IV:2 subject (Figure 2). Notably, sweating was normal in all the affected family members. This finding confirms the association of the same variant with oligodontia observed in a German family (Ruiz-Heiland et al., 2016). The amino acid substitution p.Arg289His is located in the C-terminal TNF homology domain, responsible for receptor binding at the monomer–monomer interface (Hymowitz et al., 2003). Other missense mutations affecting the same amino acid residue Arg289 (c.865C>T p.Arg289Cys, c.866G>T p.Arg289Leu), but causing different amino acid substitutions, have been reported to be associated with non-syndromic oligodontia (Lee et al., 2014; Song et al., 2009). The p.Arg289Cys (c.865C>T) variant was detected in a Chinese patient who was missing 15 teeth but showed no additional clinical signs of HED (Song et al., 2009). Moreover, p.Arg289Leu (c.866G>T) was described in a Korean family in which males were affected by severe oligodontia co-segregating with curly hair and only a few females showed hypodontia and curly hair (Lee et al., 2014).

In the present study, the variant identified in EDA is a causative mutation of HED, characterized by a recessive X-linked inheritance model. However, the disease showed variable expression, with males showing oligodontia and shape anomalies, in some cases, in association with additional symptoms. Conversely, the heterozygous females showed only hypodontia, representing an example of expressivity conditioning inheritance. Although HED is a recessive X-linked syndrome, oligodontia manifests itself as a dominant trait characterized by incomplete penetrance in heterozygous females. This appears to be consistent with the model proposing a high level of EDA-EDAR signaling for the development of human dentition, whereas other ectodermal appendages normally form in response to EDA mutations with reduced activity (Mues et al., 2010). The observed phenotypic–genotypic relationship appears to be in agreement with that described in the German family, in which oligodontia was associated with minor variants of HED (Ruiz-Heiland et al., 2016).

The novel missense variant p.Phe96Ser in EDAR was identified in family A, which showed distinctive HED symptoms. These findings strongly suggest that the EDAR c.287T>C mutation in exon 4 is the cause of the disease with autosomal dominant inheritance because of its phenotypic manifestation also in heterozygosity and family segregation.

The novel variant is located in the extracellular ligand-binding domain (aa 30-148) within the TNF receptor-like domain (TNFR2; Uniprot ID: Q9UNE0). The binding of EDAR to
trimeric EDA is mediated by this extracellular domain, while the DD is responsible for binding to its cytoplasmic signaling adaptor EDARADD. In this region, there are mostly missense mutations showing recessive inheritance. On the other hand, all the dominant mutations in EDAR described thus far are located in the exon 12 encoding the DD (Chassaing et al., 2006; Chaudhary et al., 2017; Cluzeau et al., 2011), providing further evidence that the position of variants affects the inheritance mode of HED, which varies from recessive to dominant.

However, the inheritance pattern of some mutations is not clear, in particular, for those neighboring the novel p.Phe96Ser variant. The p.Arg89His has been described in a compound heterozygous for two separate mutations (Chassaing et al., 2006; Monreal et al., 1999; Van Der Hout et al., 2008). Two reports considered this mutation to be recessive (Chassaing et al., 2006; Monreal et al., 1999), but Van der Hout et al. (Van Der Hout et al., 2008) observed very mild phenotypic expression in the heterozygous carrier of the p.Arg89His mutation.

Another EDAR mutation, close to the variant investigated in the present study, is the p.Arg98Trp variant described in an Indian family exhibiting compound heterozygosity. This mutation had been considered autosomal recessive, but an analysis of the proband’s parents suggested that it was a de novo mutation originating during spermatogenesis (Bashyam et al., 2012).

The analysis of the segregation of the p.Phe96Ser variant in family B suggests that the mutation may have a dominant-negative effect. The computational tools used in this investigation predicted a destabilizing effect of the variant on the protein structure stability. The 3D structural model showed that the amino acid residue 96 was exposed to the surface of the protein and involved in the binding with the ligand as confirmed by the docking analysis of the wild-type protein (Figure 4). On the contrary, the missense mutation p.Phe96Ser in the EDAR protein compromised the binding with the ligand.

This study provides evidence of the pathogenicity of a novel mutation in the EDAR (c.287T>C, p.Phe96Ser) and its involvement in HED. Moreover, it clarifies the role of EDA c.866G>A (p.Arg289His) in X-linked HED and tooth agenesis. These findings shed light on the relationship between the inheritance mode and phenotype of these variants, which could be helpful in genetic counseling.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
FA contributed to the design of the study, the execution of most of the experiments, the interpretation of the results, and the writing of the manuscript. DB contributed to the experimental work, the interpretation of the results, and writing of the manuscript. CS and OS contributed to the design of the study and provided the clinical information on the patients. AF designed and supervised the study, contributed to the interpretation of the results and to the writing, revising, and editing of the manuscript. MM contributed to the interpretation of the results, supervised the study, and revised the manuscript. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
All relevant data are within the paper.

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