Novel phenotype of syndromic premature ovarian insufficiency associated with *TP63* molecular defect.

Short running title:

TP63 associated syndromic POI

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Abstract:

There is growing evidence that *TP63* is associated with isolated as well as syndromic premature ovarian insufficiency (POI). We report two adolescent sisters diagnosed with undetectable ovaries, uterine hypoplasia, and mammary gland hypoplasia. A novel paternally inherited nonsense variant in *TP63* (NM_003722.4 c.1927C>T,p.(Arg643*)) in exon 14 was identified by exome sequencing. One of the syndromes linked to *TP63* is Limb-Mammary syndrome (LMS), an autosomal dominant inherited disorder characterized by ectrodactyly, hypoplasia of mammary-gland and nipple, lacrimal duct stenosis, nail dysplasia, dental anomalies, cleft palate and/or cleft lip and absence of skin and hair defects. The *TP63* variant segregated with symptoms of LMS in the family, however no affected individual had limb defects. The phenotype reported here represents a novel syndromic phenotype associated with *TP63*. Reported cases with *TP63* associated POI are reviewed.

Key words: TP63, premature ovarian insufficiency, mammary hypoplasia, uterine hypoplasia, Limb Mammary Syndrome

Introduction:

Limb-Mammary syndrome (LMS) is an autosomal dominantly inherited *TP63* associated syndrome characterized by ectrodactyly, hypoplasia of mammary-gland and nipple, and without skin- and hair defects [1]. Lacrimal duct stenosis is seen in approximately half of the patients, and ectodermal defects (nail dysplasia and dental anomalies), and cleft palate and/or cleft lip is also reported [1]. LMS is caused by heterozygous variants located in either N-terminal (exon 4) or C-terminal (exon 13 and 14) regions of the *TP63* gene [2-6]. Variants in *TP63* have also been linked to other syndromes such as ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC), Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), acro-dermato-ungual-lacrimal-tooth syndrome (ADULT), and isolated phenotypes as isolated split hand/foot malformation (SHFM4), and isolated cleft lip and or cleft palate [2,7].

TP63 is located at 3q28 and encodes the p63 transcription factor. The use of two alternative promoters result in the two major isoforms TA and ΔN. ΔN lacks the TA specific exons 1-3 and is without the specific transactivation domain seen in the TA isoform. Alternative splicing of both TA and ΔN give rise to additional C-terminal isoforms (α,β,γ,δ,ε) [8]. The p63 transcription factor is highly expressed in the skin, and is a major regulator of epidermal development, but is also expressed in various other tissues [9,10]. In epithelial cells and tissues it seems that the predominant isoform is ΔN [9,11-13]. The TA isoform is expressed in other tissues such as cochlea, late-stage myogenesis and in cardiomyocyte development [14-16], and it is the only isoform expressed in oocytes where it is involved in controlling apoptosis in response to DNA damage [17-19].

Ovarian development involves many processes including mitotic proliferation of primordial germ cells, meiosis and creation of primordial follicles [20]. Multiple genes are involved in syndromic and non-syndromic premature ovarian insufficiency and gonadal dysgenesis [20]. The overall term premature ovarian insufficiency (POI) will be used throughout this paper as recommended in the ESHRE guideline from 2016, and covers POI with primary as well as secondary amenorrhea[21]. There is increasing evidence that *TP63* variants play a role in POI[6,22-24].

We present a family with features consistent with a LMS phenotype but without limb anomalies, of which the two affected sisters presented with undetectable ovaries, uterine hypoplasia, and mammary gland and nipple hypoplasia. A novel nonsense variant in *TP63* (NM_003722.4

c.1927C>T,p.(Arg643*)) in exon 14 was identified by exome sequencing, and segregated with symptoms of LMS in the family.

Materials and Methods:

The pedigree of the family of Danish ancestry is seen in figure 1. Clinical information is presented in table 1 and appendix 1. The sisters III-2 and III-4 both had undetectable ovaries, uterine hypoplasia, and mammary gland and nipple hypoplasia, and were the primary focus of the genetic investigation.

Methods:

A tetra-based whole-exome sequencing approach was undertaken. DNA from individuals III-2 and III-4 and parents (II-5 and II-6) were subjected to exome capture using NimbleGen SeqCap EZ MedExome (Roche), followed by sequencing on an Illumina NextSeq550 to a mean coverage of 130x, with 95% of targeted bases covered with minimum 20x coverage. Raw reads were aligned using the Burrows-Wheeler Alignment tool (BWA-MEM) v. 0.7.15 [25] and the GATK Best Practice pipeline v. 3.8–0 was used for variant calling [26]. Annotation and filtering of variants was performed using VarSeq 2.1.0 (Golden Helix). The shared rare variants were ranked (PhoRank) according to the Human Phenotype Ontology term HP:0000786: primary amenorrhea. The variants with highest rank were classified (See suppl. Material)

Bidirectional Sanger sequencing of *TP63* (reference NM_003722.4) and *FGFR2* (reference NM_022970.3) was performed according to standard procedures.

Results:

Exome sequencing showed heterozygosity for a novel nonsense variant *TP63*(NM_003722.4):c.1927C>T,p.Arg643* in the two sisters and in the father. The variant was not present in the gnomAD database (December 2019), where the probability of loss of function intolerance score (pLI score) was 1 for *TP63* predicting the gene to be intolerant for loss of function mutations. The variant was located in exon 14 within the transactivation inhibitory domain (TI

domain) and was predicted to cause a truncated protein. Segregation analysis showed that the *TP63* variant was also present in individuals II:1, III:3 and III:5. Based on the pathogenicity evidence given under the ACMG standards and guidelines, it was classified as pathogenic. A missense variant in *FGFR2* in exon 9 (c.1280G>A,p.(Arg427Lys)) was also identified in the two sisters and in the father. The variant was not present in the gnomAD database. Established in silico prediction tools predicted the missense variant in *FGFR2* to be damaging. Segregation analysis for the *FGFR2* variant showed that this variant was also present in III-5, II-1, II-3 and I-2. According to the pathogenicity evidence given under the ACMG standards and guidelines, this variant would be classified as of uncertain significance. See supplementary material for more details.

Discussion:

We present two sisters with undetectable ovaries, hypergonadotropic hypogonadism, uterine hypoplasia, mammary gland and nipple hypoplasia, and with normal hands and feet. With estrogen treatment, the size of the uterus increased to normal or near normal size for age in both sisters, and they acquired normal bleeding periods. We therefore consider the uterine hypoplasia to be secondary to the ovarian insufficiency. One sister had lacrimal duct atresia. They both inherited a nonsense variant in *TP63* from their father, and segregation analysis showed that the male relatives who also had the variant presented features such as nipple hypoplasia, lacrimal duct atresia, dental anomalies, nail dysplasia, cleft palate, or anorectal malformation (Table 1, Fig 1). No other female family members had the *TP63*-variant. The features in the affected individuals were compatible with Limb mammary syndrome (LMS) except that none had limb anomalies. Since limb anomalies are very frequent in LMS[1], this is remarkable. Nipple anomalies (6/6) and lacrimal duct atresia (4/6) were the most frequent features among the affected individuals, which is in concordance with previously reported frequencies of LMS [1]. The genitalia of the affected males were not examined, but the two adult men had both reproduced. ADULT syndrome is the only other *TP63* related syndrome with mammary gland hypoplasia. However, cleft palate is not a frequent feature of ADULT syndrome, and skin- and hair defects, which are characteristic for ADULT syndrome, are not part of the phenotype in the family presented, making the ADULT diagnosis unlikely [4]. The *TP63* variant is furthermore located in exon 14 in the transactivation inhibitory domain, where other

variants causing LMS are previously reported [3,5]. The family thus represents a novel TP63 associated LMS-like phenotype without limb anomalies, and with POI.

TP63 has multiple roles in the development of the lower female reproductive tract. This involves vaginal epithelial differentiation from Müllerian epithelium, cloacal septation, and modeling of external genitalia [27]. Studies have shown that TAp63 is expressed in oocytes where it is involved in controlling apoptosis in response to DNA damage and thereby maintaining genomic integrity of oocytes [17-19]. It has been suggested that *TP63* truncating variants might disrupt *TP63* inhibition, thereby resulting in uncontrolled oocyte death [22].

There is increasing evidence that *TP63* is associated with POI as recently reviewed by Tucker et al. [22]. Syndromic *TP63* associated POI has previously been described in a patient with LMS and hypergonadotropic hypogonadism with undetectable ovaries and uterus [6]. Furthermore, in a family with Rapp Hopkin Syndrome, POI was seen together with other features of early aging [23]. Isolated POI was reported in two teenagers with a paternally inherited intragenic duplication in *TP63* [24], and in two patients with nonsense variants in *TP63* exon 14, predicted to cause a truncated protein [22] (figure 2). The phenotypes associated with POI and *TP63* variants are thus diverse and includes isolated POI as well as syndromic POI of different types: Rapp Hopkin Syndrome, LMS, and a novel LMS-like phenotype without limb anomalies as seen in our family. Apart from the intragenic duplication, all *TP63*-variants reported associated with POI so far are in the C-terminal region of *TP63* supporting that this region is especially important for ovary development and function.

We initially wondered if the *FGFR2* variant could affect the genital phenotype in the probands. However, the *FGFR2* variant but not the *TP63* variant, was identified in the paternal aunt (II-3) who had normal female genitalia as well as normal breast development. Therefore, the *FGFR2* variant is not an obvious cause for the ovarian agenesis of the affected individuals. We cannot exclude, that the *FGFR2* variant had some effect on the uterine hypoplasia, but the fact that the size of the uterus normalized/nearly normalized in the two sisters after estrogen treatment, suggests that the uterus hypoplasia was most likely secondary to estrogen deficiency.

In conclusion we have reported a novel *TP63* associated phenotype with many similarities to LMS, but without limb defects, and with POI in the affected females. Phenotypes in individuals with POI and *TP63* variants are diverse as some have isolated POI, while others have syndromic POI. In order to identify the syndromic cases, thorough phenotyping is required, as some features may be subtle as demonstrated by the family reported here. Our study supports the growing evidence that *TP63* is important in oocyte function and development of the ovaries. We also show that estrogen treatment of young women with variants in *TP63* and inner genital anomalies might enable them to grow a normal sized uterus and to achieve bleeding periods.

References

1. van Bokhoven H, Jung M, Smits APT, et al. Limb Mammary Syndrome: A New Genetic Disorder with Mammary Hypoplasia, Ectrodactyly, and Other Hand/Foot Anomalies Maps to Human Chromosome 3q27. *The American Journal of Human Genetics.* 1999;64(2):538-546.
2. Rinne T, Brunner HG, van Bokhoven H. p63-Associated Disorders. *Cell Cycle.* 2007;6(3):262-268.
3. Rinne T, Hamel B, Bokhoven Hv, Brunner HG. Pattern of p63 mutations and their phenotypes—update. *American Journal of Medical Genetics Part A.* 2006;140A(13):1396-1406.
4. van Bokhoven H, Brunner HG. Splitting p63. *American journal of human genetics.* 2002;71(1):1-13.
5. van Bokhoven H, Hamel BC, Bamshad M, et al. p63 Gene mutations in eec syndrome, limb-mammary syndrome, and isolated split hand-split foot malformation suggest a genotype-phenotype correlation. *American journal of human genetics.* 2001;69(3):481-492.
6. Guazzarotti L, Caprio C, Rinne TK, et al. Limb-mammary syndrome (LMS) associated with internal female genitalia dysgenesia: A new genotype/phenotype correlation? *American Journal of Medical Genetics Part A.* 2008;146A(15):2001-2004.
7. Khandelwal K, Boogaard Mvd, Mehrem SL, et al. Deletions and loss-of-function variants in TP63 associated with orofacial clefting. *European Journal of Human Genetics.* 2019;27(7):1101-1112.
8. Soares E, Zhou H. Master regulatory role of p63 in epidermal development and disease. *Cellular and molecular life sciences : CMLS.* 2018;75(7):1179-1190.
9. Dellavalle RP, Egbert TB, Marchbank A, Su L-J, Lee LA, Walsh P. CUSP/p63 expression in rat and human tissues. *Journal of Dermatological Science.* 2001;27(2):82-87.
10. Osada M, Ohba M, Kawahara C, et al. Cloning and functional analysis of human p51, which structurally and functionally resembles p53. *Nature Medicine.* 1998;4(7):839-843.
11. Pellegrini G, Rama P, Matuska S, et al. Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. *Regenerative Medicine.* 2013;8(5):553-567.
12. Zhao Q, Liu S, Zhang H, et al. Spatiotemporal Expression of p63 in Mouse Epidermal Commitment. *International journal of molecular sciences.* 2015;16(12):29542-29553.
13. Romano R-A, Smalley K, Magraw C, et al. ΔNp63 knockout mice reveal its indispensable role as a master regulator of epithelial development and differentiation. *Development (Cambridge, England).* 2012;139(4):772-782.
14. Terrinoni A, Serra V, Bruno E, et al. Role of p63 and the Notch pathway in cochlea development and sensorineural deafness. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(18):7300-7305.
15. Cefalù S, Lena AM, Vojtesek B, et al. TAp63gamma is required for the late stages of myogenesis. *Cell cycle (Georgetown, Tex).* 2015;14(6):894-901.
16. Rouleau M, Medawar A, Hamon L, et al. TAp63 Is Important for Cardiac Differentiation of Embryonic Stem Cells and Heart Development. *STEM CELLS.* 2011;29(11):1672-1683.

17. Suh E-K, Yang A, Kettenbach A, et al. p63 protects the female germ line during meiotic arrest. *Nature.* 2006;444:624.
18. Deutsch GB, Zielonka EM, Coutandin D, et al. DNA damage in oocytes induces a switch of the quality control factor TAp63α from dimer to tetramer. *Cell.* 2011;144(4):566-576.
19. Crum CP, McKeon FD. p63 in epithelial survival, germ cell surveillance, and neoplasia. *Annual review of pathology, 2010, Volume 5.* 2010;5:349.
20. Yatsenko SA, Rajkovic A. Genetics of human female infertility. *Biology of reproduction.* 2019;101(3):549-566.
21. Webber L, Davies M, Anderson R, et al. ESHRE Guideline: management of women with premature ovarian insufficiency. *Human Reproduction.* 2016;31(5):926-937.
22. Tucker EJ, Jaillard S, Grover SR, et al. TP63-truncating variants cause isolated premature ovarian insufficiency. *Human Mutation.* 2019;0(0).
23. Holder-Espinasse M, Martin-Coignard D, Escande F, Manouvrier-Hanu S. A new mutation in TP63 is associated with age-related pathology. *European journal of human genetics : EJHG.* 2007;15(11):1115-1120.
24. Bestetti I, Castronovo C, Sironi A, et al. High-resolution array-CGH analysis on 46,XX patients affected by early onset primary ovarian insufficiency discloses new genes involved in ovarian function. *Human Reproduction.* 2019;34(3):574-583.
25. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *eprint arXiv:13033997.* 2013:arXiv:1303.3997.
26. Van der Auwera GA, Carneiro MO, Hartl C, et al. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. *Current protocols in bioinformatics.* 2013;43(1110):11.10.11-11.10.33.
27. Ince TA, Cviko AP, Quade BJ, et al. p63 Coordinates anogenital modeling and epithelial cell differentiation in the developing female urogenital tract. *The American journal of pathology.* 2002;161(4):1111-1117.

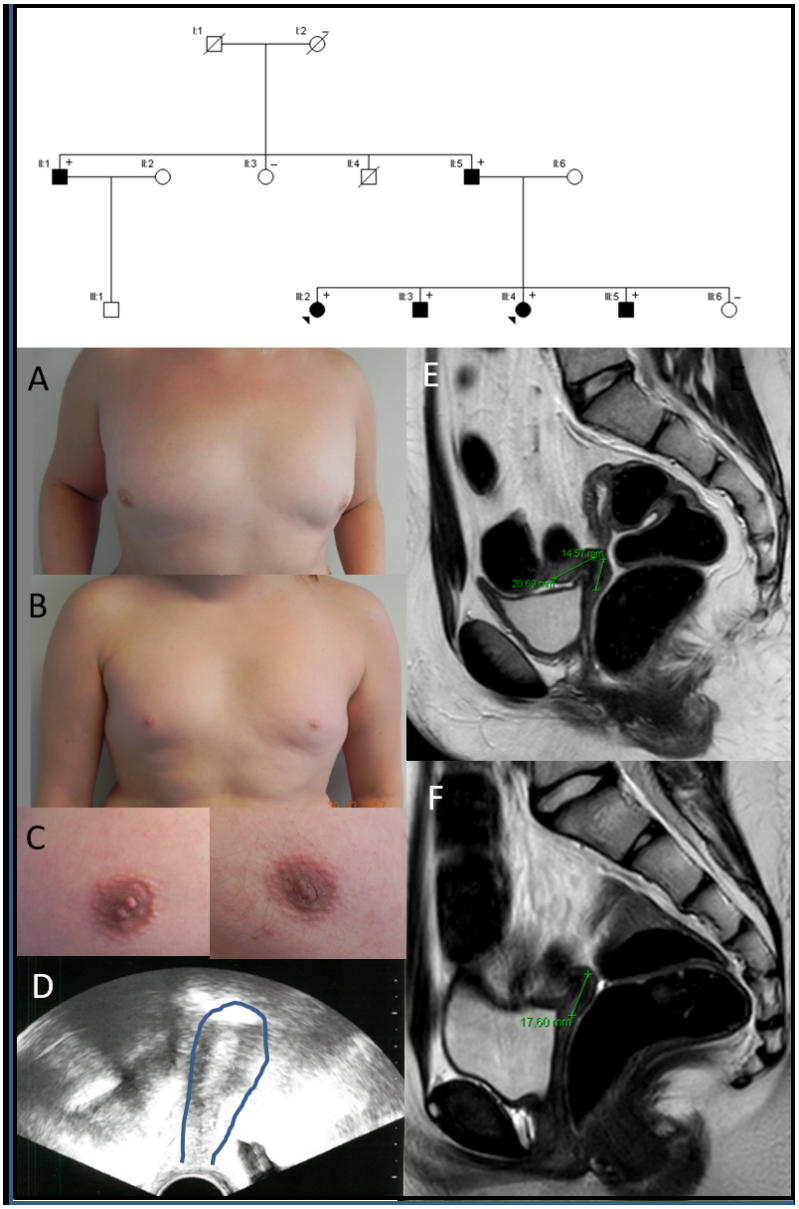

Figure 1.Pedigree: filled symbols indicate affected individuals, +/- indicate presence or absence of the TP63 variant c.1927C>T,p.Arg643*. The two sisters with ovarian agenesis are individuals III-2 and III-4. All individuals with the TP63 variant had some signs of Limb-mammary syndrome, however none had limb defects. Photos show mammary hypoplasia and nipple hypoplasia and long distance between nipples in individuals III-2 and III-4 (A, B), split nipple and hypoplastic nipple in individual II-5 (C). D: US-scan showing nearly normal sized uterus for age (2x5 cm) after estrogen treatment (uterus is marked with a blue line). E, F: MR-scan showing hypoplastic uterus before estrogen treatment in individuals III-2 and III-4.

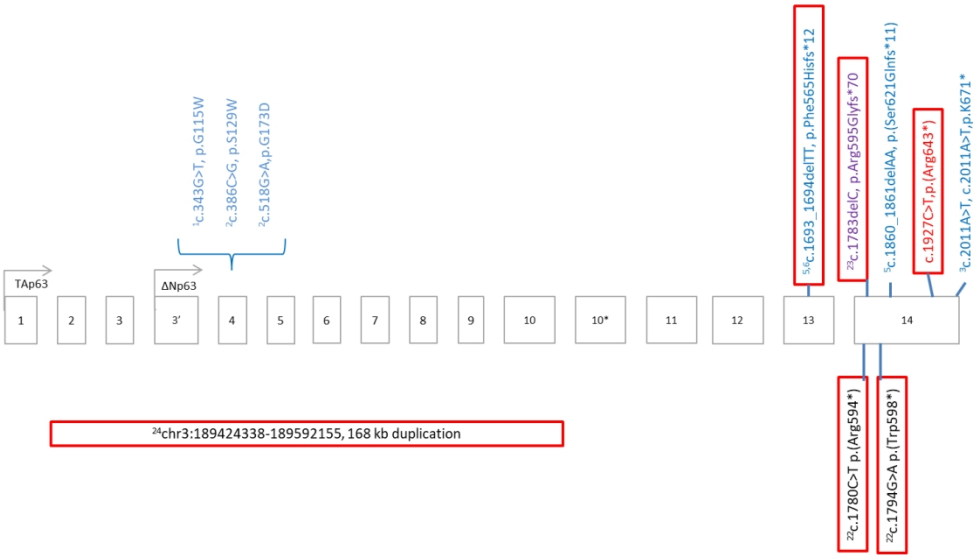

Figure 2.Schematic figure of variants in TP63 from literature associated with Limb Mammary Syndrome (LMS) and/or premature ovarian insufficiency, POI (syndromic or isolated), according to accession no. NM_003722.4 . Other TP63 phenotypes are not included. Blue text indicates LMS, purple text indicates Rapp Hopkin syndrome, and the present family with an LMS like phenotype, but without limb anomalies is in red text. Black text indicates isolated POI. All cases with POI are in a red box. Numbers in superscript refers to the reference list.

	II-1	II-5	III-2	III-3	III-4	III-5	Features known from Limb Mammary syndrome
Sex	M	M	F	M	F	M	
Age	49	42	22	19	17	13	
Mammary hypoplasia	NR	NR	+	NR	+	NR	+
Nipple abnormality	Supernummary	Split	Small nipples, large distance	Bean shaped	Small	Large distance	+
Lacrimal duct atresia	+	-	+	+	-	+	+
Dental symptoms	NI	Agenesis of 12 & 22 (& 18,28,38,48) Severe caries Moderate-severe attrition	Caries decay Moderate-severe attrition	NI	Moderate attrition	-	+
Nail dysplasia	-	5th toe bilateral	-	-	-	-	+
Cleft lip +/- cleft palate	-	-	-	+	-	-	+
Split hand/foot or syndactyli	-	-	-	-	-	-	+
Ovarian agenesis	NR	NR	+	NR	+	NR	Reported in one case[6]
Uterine hypoplasia†	NR	NR	+	NR	+	NR	Reported in one case[6]
Other symptoms	Anterior placed anus. Obstr. sleep apnea ‡Growth hormone deficiency	Ganglion cysts Sleep apnea		Strabismus			