Elena Tucker ORCID iD: 0000-0002-6380-5100

***TP63* truncating variants cause isolated premature ovarian insufficiency**

Elena J. Tucker^{1,2}, Sylvie Jaillard^{1,3}, Sonia R. Grover^{1,2,4}, Jocelyn van den Bergen¹,
Gorjana Robevska¹, Katrina M. Bell¹, Simon Sadedin^{1,2}, Chloe Hanna^{1,4}, Jérôme Dulong⁵,
Philippe Touraine⁵, Andrew H. Sinclair^{1,2}

¹Murdoch Children's Research Institute, Melbourne, VIC Australia, 3052

²Department of Paediatrics, University of Melbourne, Melbourne, VIC Australia,
3052

³Univ Rennes, CHU Rennes, INSERM, EHESP, IRSET (Institut de recherche en
santé, environnement et travail) – UMR_S 1085, F-35000 Rennes, France

⁴Department of Paediatric and Adolescent Gynaecology, Royal Children's
Hospital, Melbourne, VIC Australia, 3052

⁵AP-HP, Sorbonne University Medicine, Department of Endocrinology and
Reproductive Medicine, Centre de référence des maladies endocriniennes rares de
la croissance et du développement; Centre des pathologies Gynécologiques rares,
Paris, France

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ABSTRACT

Premature ovarian insufficiency involves amenorrhea and elevated follicle stimulating hormone before age 40, and its genetic basis is poorly understood. Here we study 13 POI patients using whole exome sequencing. We identify *PREPL* and *TP63* causative variants, and variants in other potentially novel POI genes. *PREPL* deficiency is a known cause of syndromic POI, matching the patients' phenotype. A role for *TP63* in ovarian biology has previously been proposed but variants have been described in multi-organ syndromes, and not isolated POI. One patient with isolated POI harbored a *de novo* nonsense *TP63* variant in the terminal exon, and an unrelated patient had a different nonsense variant in the same exon. These variants interfere with the repression domain while leaving the activation domain intact. We expand the phenotypic spectrum of *TP63*-related disorders, provide a new genotype:phenotype correlation for *TP63* and identify a new genetic cause of isolated POI.

Key words: Premature ovarian insufficiency, TP63, PREPL, POI cohort, whole-exome sequencing

MAIN TEXT

Premature ovarian insufficiency (POI) occurs when a woman experiences amenorrhea associated with elevated follicle stimulating hormone (FSH) before the age of 40. POI can be secondary to chemotoxic/cancer therapy, physical trauma or ovarian surgery, infection or autoimmunity, however, many cases are sporadic and unexplained. There is a clear genetic component to POI, however, it is highly heterogeneous (Tucker, Grover,

Bachelot, Touraine, & Sinclair, 2016). POI-related genes have diverse roles including metabolism, folliculogenesis, gonadogenesis, oogenesis, DNA damage repair, apoptosis, hormone signaling, autoimmunity and more. The known genes explain only a minority of cases and our understanding of POI genetics is far from complete. In addition to the genes known to be involved in human POI, there are hundreds of candidate genes identified by studies in model organisms, by genome-wide association studies of age at natural menopause or POI, implicated by the identification of copy number variants (CNVs) or single nucleotide variants (SNVs) in POI patients, by *in vitro* studies or a relationship to known POI genes.

In order to provide molecular diagnoses to patients and to identify new genes underpinning this incompletely understood condition, we recruited a diverse cohort of 13 patients through the paediatric gynaecology department at the Royal Children's Hospital, Melbourne. Clinical information is included in Supp. Table S1. The diversity of our cohort reflects clinical reality, with variability in age of onset and associated features. All patients had POI, defined by menstrual disturbance and elevated FSH (>20 mIU/mL) measured twice at least one month apart as per the European Society of Human Reproduction (ESHR) guidelines (ESHR et al., 2016). Karyotyping was performed to confirm 46,XX chromosomal complement, and to exclude patients with causal chromosomal rearrangements. All included cases were negative for *FMR1* premutation and negative for ovarian auto-antibodies. Written informed consent was obtained from all participants and procedures were in accordance with the ethical standards of the Human Research Ethics Committee of the Royal Children's Hospital, Melbourne. Familal DNA

was obtained from 8 patients, with pedigrees depicted in Supp. Figure S1. DNA underwent high-density microarray analysis and whole exome sequencing, followed by gene-centric and variant-centric data analysis. Detailed methods are provided in supplementary materials, and genes prioritized for gene-centric analysis are listed in Supp. File S1. Sequencing statistics including mean depth of coverage, are provided in Supp. File S2. Reported SNVs are submitted to ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>).

We were able to identify the genetic cause of POI in Patient 8 who presented with primary amenorrhea and had a diagnosis of isolated POI. WES of the patient and both her parents revealed 572 high quality coding variants in the patient. Ten of these were within candidate POI genes, although based on their segregation, pathogenicity predictions/conservation scores, MAF and/or the lack of a second variant in the same gene, these did not represent strong causal candidates.

Variant-centric analysis revealed 29 high priority variants, none of which showed recessive-type inheritance, and 10 of which had a potential dominant inheritance, being absent in the unaffected mother (presence in the father was permissible given POI is female-specific). The strongest candidate variant was a *de novo* nonsense variant in *TP63* (MIM# 603273). The NM_003722.4 (*TP63*): c.1780C>T p.(Arg594Ter) variant is within the terminal exon of *TP63* and is predicted to introduce a premature stop codon. Its location in the final exon means the resulting transcript likely escapes nonsense-mediated decay, and a truncated p63 protein is predicted. *TP63* has at least six different isoforms (Figure 1A) and variants have been described in congenital multiorgan syndromes

(Figure 1B). The c.1780C>T p.(Arg594Ter) variant falls within the sterile alpha motif (SAM) domain and truncates TP63 before the transactivation inhibitory domain. Using ACMG guidelines (ACMG, 2015), this variant was curated as pathogenic (Supp. Table S2).

To investigate the contribution of *TP63* to POI, we screened the terminal exon of *TP63* for variants by Sanger sequencing in an additional 107 POI patients. This revealed a different *TP63* C-terminal truncating variant in an unrelated patient (Figure 1C), who also presented with primary amenorrhea and had a diagnosis of isolated POI. She carried a heterozygous NM_003722.4 (*TP63*): c.1794G>A p.(Trp598Ter) variant, also considered pathogenic by ACMG guidelines given it was a null-type variant in a known disease gene, was absent from controls and was associated with the same phenotype as the original patient who carried a pathogenic variant in this gene (Supp. Table S2). We performed WES on this patient and verified that no other likely causative variant was present.

Other *TP63* variants detected by Sanger sequencing or WES were not considered likely pathogenic. One patient carried an intronic NM_003722.4 (*TP63*): c.1747-34T>C variant, which was not detected in public databases and fell within a predicted branch site region for RNA splicing, however, was predicted to enhance its strength as a branch site (<http://www.umd.be/HSF3/>). Two patients carried a common SNP in the 3' UTR, NM_003722.4 (*TP63*): c.2043+45C>T. No other *TP63* variants were detected in the remaining 103 patients.

We failed to detect the *TP63* causative variant in Patient 8 using our gene-centric protocol. Although this gene had not previously been recognized as a POI candidate by us, literature search confirmed a clear potential for involvement. The longest *TP63* isoform, referred to as *TAp63α*, is almost exclusively expressed in female germ cells and is considered a “supervisor and executioner” that ensures germ cell fidelity (Crum & McKeon, 2010). The timing of *TAp63α* expression is consistent with a role in the management of DNA damage of oocytes. It is not expressed during non-reductive DNA replication, during which homologous recombination occurs and DNA double strand breaks are permissible. As oocytes enter dictyate arrest, *TAp63α* expression is initiated and maintained until oocytes are recruited for ovulation (Suh et al., 2006). During this time, *TAp63α* induces apoptosis in oocytes that have undergone DNA damage (Gonfloni, 2010; Suh et al., 2006). Despite this important role in protection of oocytes, *TAp63* null mice have no ovarian phenotype in normal conditions (Suh et al., 2006; Yang et al., 1999). The role of *TAp63*, however, becomes evident under genotoxic stress. Irradiated wild-type mice experience a severe and specific loss of small primordial follicles. In contrast, the same radiation dose does not lead to loss of oocytes in *TAp63* null mice, confirming that *TAp63* is essential for oocyte death in response to DNA damage (Suh et al., 2006).

Pathogenic variation in *TP63* is usually associated with autosomal dominant congenital malformation of multiple organs, particularly those of ectodermal origin, such as hair, teeth and skin. These include ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome (MIM# 604292), acro-dermato-ungual-lacrima-tooth (ADULT)

syndrome (MIM# 103285), ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome (MIM# 106260), Hays-Wells syndrome (MIM# 106260), Rapp-Hodgkin syndrome (MIM# 129400) and limb-mammary syndrome (MIM# 603543) (Rinne, Hamel, van Bokhoven, & Brunner, 2006). Two TP63-related cases are described in the literature with POI as a component of their syndromic phenotype (Guazzarotti et al., 2008; Holder-Espinasse, Martin-Coignard, Escande, & Manouvrier-Hanu, 2007). No syndromic malformations were noted in the two patients with *TP63* nonsense variants detected by our study. All variants causing TP63-related POI affect the C-terminal transactivation inhibition domain (Figure 1B), but the syndromic cases are associated with frameshift variants in contrast to the isolated POI cases described here with nonsense variants. Phenotypic and genetic comparison of the patients with syndromic POI described in the literature and the two patients identified by this study are provided in Supp. Table S3.

Taken together, these cases clearly demonstrate the importance of the C-terminal transactivation inhibition domain of TP63 to oocyte function. In non-stressed oocytes, TAp63 α exists as an inactive dimer (Deutsch et al., 2011). This conformation relies on the interaction between the N-terminal transactivation domain and the C-terminal transactivation inhibition domain (Deutsch et al., 2011; Serber et al., 2002; Straub et al., 2010). DNA damage and oocyte stress lead to the phosphorylation of TP63, disrupting these inhibitory interactions, allowing TP63 to tetramize. This active tetramer is then able to bind DNA, activate transcription and promote oocyte destruction (Deutsch et al., 2011). The TP63 truncation variants detected in our patients likely disrupt TP63

inhibition, leading to uncontrolled oocyte death and subsequent POI.

Genotype:phenotype correlation for *TP63* has been investigated previously, establishing that variants causing the life-threatening AEC syndrome tend to be C-terminal frameshift variants that promote protein aggregation (Russo et al., 2018). In contrast, variants causing the milder EEC syndrome tend to be missense variants within the DNA binding domain and are not associated with protein aggregation. Frameshift variants that introduce aggregation signals in the C terminus of TP63, not only promote self-aggregation, but also cause aggregation with the related TP73 (Russo et al., 2018), thereby impacting multiple developmental processes and having a broader phenotypic consequence. It is possible that the frameshift variants in the cases of TP63-related syndromic POI described in the literature introduce such aggregation signals, but the nonsense variants in the two patients described here, do not, leading to their milder phenotype of isolated POI.

A second genetic diagnosis was achieved in our POI cohort, for two sisters (Patient 3 and 3S) diagnosed post-natally with an undefined neuromuscular condition. They both experienced hypotonia and delayed mobility in infancy, and primary amenorrhea in adolescence. Slight dysmorphism was noted (mainly high arched palate), as well as short stature, nasal speech, mild learning difficulties and intermittent ptosis. Gene-centric analysis focusing on candidate genes did not identify any likely causative variants, but variant-centric analysis revealed an apparently homozygous NM_006036.4 (*PREPL*): c.881_955delinsTTTTA p.(Lys294IlefsTer8) variant within the *PREPL* gene (MIM# 609557) shared by the sisters. The patients' mother was heterozygous for the same

variant, whereas no likely pathogenic *PREPL* variant was detected in the patients' father (Supp. Figure S2A). The lack of paternal variant indicated a potential deletion had been missed by WES. We performed high density microarrays which showed that, as suspected, both sisters carried a heterozygous deletion, arr[GRCh37] 2p21(44505149_44579929)x1. By PCR and sequencing, we determined the base pair resolution of the deletion to be seq[GRCh37] del(2)(2p21) chr2:g.44504991_44580502del, and confirmed that the 75.5kb deletion was paternally inherited (Supp. Figure S2B). The heterozygous deletion spans the last 9 exons of the *SLC3A1* gene and the last 13 exons of the *PREPL* gene (Supp. Figure S2C). Homozygous deletion of this region, which encompasses *SLC3A1* as well as *PREPL*, is a well-established cause of hypotonia-cystonuria (MIM# 606407), a condition with clinical overlap with the sisters' phenotype. The combination of the large deletion and the novel indel affecting only *PREPL*, led to the diagnosis of isolated *PREPL* deficiency. At the time of genetic diagnosis, isolated *PREPL* deficiency (MIM# 616224) had only been described once in the literature with no long-term follow-up of the affected patient (Regal et al., 2014). Recently, endocrine involvement, including hypergonadotropic hypogonadism (POI), has been described in *PREPL* deficient patients (Regal et al., 2018). Scoring the *PREPL* variants using ACMG guidelines (ACMG, 2015), reveals these null-type compound heterozygous variants that segregate with disease in the family, are pathogenic (Supp. Table S2). This enabled a clarification of the patients' undefined neuromuscular condition thereby improving patient management, and confirms the involvement of endocrine dysfunction and POI in this recently characterized condition.

We were unable to provide conclusive diagnoses to the remaining 11 patients in our cohort, however, data analysis identified intriguing candidate variants, as summarized in Table 1 and Supp. File S3. Detailed discussion of candidate variants is provided in supplementary materials. These candidate variants are found in DNA damage repair genes (*RAD50*, *MRE11A*, *RAD51C*, *NEK8*, *MCM9*, *RAD51C*), genes involved in transcriptional function and downstream genome stability (*ERCC6L2*, *CCNT2*), genes involved in DNA replication (*CDC7*, *DDI1*), chromosomal alignment (*CENPI*, *REC8*), chromosomal segregation (*CAMSAP2*, *SSH1*, *CROCC1*), and genes involved in folliculogenesis (*HERC1*, *GREM1*, *ESRRG*, *ESRRB*).

Interestingly, the two genetic diagnoses achieved in our small cohort, did not harbour causative variants in known and well-established POI genes. They were therefore filtered out in the first phase of analysis, which was “gene-centric” and focused on variants within genes of a pre-defined list (Supp. File S1). Indeed, several lists of POI-related genes have been published and/or used in next-generation sequencing studies (Arboleda et al., 2013; Bramble et al., 2016; Patino et al., 2017; Qin, Jiao, Simpson, & Chen, 2015; Tucker et al., 2016). A list of suspected POI genes is difficult to compile, and there is a need to compromise between the number of candidate genes included and the ease of downstream analysis. Including only genes with evidence linking them directly to human POI may significantly reduce the number of variants that need to be assessed for their potential involvement, but may cause many variants to be overlooked in genes that are not so well-linked to POI. In contrast, a comprehensive candidate list that includes any gene with a direct or indirect link to ovarian function does not so effectively reduce the

number of variants of interest and the effort of downstream analysis. In keeping with this, all reported lists have a varied capture of POI-genes, and there has been only modest success using panels for the genetic analysis of patients with POI. Recognizing the shortcomings of stringent criteria for analysis, WES data from published POI cohorts has been re-analysed to identify previously-missed diagnoses in *KHDRBS1*, *ATG7* and *ATG9A* (Delcour et al., 2018; Wang et al., 2017). Our gene list contained 482 genes which had a predicted role in POI based on known variants in human cases, relevant phenotypes in mice with gene disruption, genetic associations in POI cohorts, and/or *in vitro* studies (Supp. File S1). We, too, acknowledge the shortcomings of our gene list and modified our analysis protocol to investigate three types of variants: 1) any variant in a candidate POI gene, 2) high priority variants in any gene, 3) recessive-type variants in any gene. This enabled the genetic diagnosis of patients with causative *TP63* and *PREPL* variants, neither of which was in our candidate gene list (nor those published by others) despite a potential role in human ovarian biology.

Despite the successful genetic diagnosis in this small POI cohort (two cases of 13 analyzed), the majority of cases remain unexplained and warrant further investigation. As presented in Table 1 and Supp. File S3 and discussed in supplementary materials, most patients harbor multiple variants of interest. It is possible that multiple variants cause POI in a multigenic manner. An example of potential oligogenic inheritance is demonstrated in Patient 7 who carries a likely damaging variant in *MCM9* as well as *BMPR1B*. The particular *MCM9* variant has previously been reported in at least 3 other POI patients, one of whom also harbours a likely damaging *BMPR1B* variant (Desai et al., 2017; Patino

et al., 2017). The repeated detection of this *MCM9* variant in POI patients, could be due to chance or could reflect genetic predisposition to POI, with POI only manifesting if another gene, such as *BMPRI1B*, is also compromised.

The selection of variants for functional validation requires careful consideration. It is important to sequence familial DNA, when available, as the inheritance pattern can inform the likelihood of causation. The sequencing of larger cohorts enabling the recognition of multiple patients and families affected by variants in the same gene will further aid prioritization of variants for functional validation. The experiments required for functional validation are gene-dependent and could involve DNA damage repair assays, reporter gene assays, gene knockdown in cell lines or animal models, and more. The variants detected and discussed in our patient cohort, will provide insight for future studies into the genetic basis of POI.

In summary, we have performed WES on a small cohort of 13 POI patients providing molecular diagnoses to two families, one with pathogenic *PREPL* variants and one with a pathogenic *TP63* variant. We have established that C-terminal truncation variants of *TP63* can cause isolated POI, expanding the phenotypic spectrum of the TP63-associated syndromes, providing a new genotype:phenotype correlation for TP63 variants, and expanding the knowledge of the genetic cause of isolated POI. The cause of POI in the remaining patients remains elusive but many interesting candidates were identified in genes involved in DNA damage repair, transcription, DNA replication, chromosomal alignment and segregation, as well as folliculogenesis. These variants will be the focus of

future studies, as will be the sequencing of larger POI cohorts to identify additional affected families.

Author Contribution

EJT, SJ, SG, PT and AHS conceived and designed the study. EJT, SJ, SG, CH, JB, GR acquired the data. EJT, SJ, KB, SG, PT and AHS analysed and interpreted the data. EJT, SJ, SG, CH, JB, GR, KB, JB, PT and AHS wrote and/or revised the manuscript.

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Conflict of interests

The authors declare no conflicts of interest.

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Table 1: Variants of interest. For further information, see Supp. File S3 and supplementary materials

ID	Phenotype	Gene	RefSeq	Variant
Patient 1	POI, facial palsy, ear deformity, deafness	<i>ERCC6L2</i>	NM_020207.4	c.2189delG p.(Gly730AspfsTer50)
		<i>ESRRG</i>	NM_001243518.1	c.1103A>G p.(Tyr368Cys)
		<i>ESRRB</i>	NM_004452.3	c.3G>A p.(Met1?)
Patient 2	POI, Wilm's tumour, multiple sclerosis	<i>CDC7</i>	NM_001134419.1	c.683_689delCCCACAT p.(Ser228Ter)
		<i>DDI1</i>	NM_001001711.2	c.692_714dup p.(Gly239Ter)
Patient 3*	POI, undefined neuromuscular condition	<i>PREPL</i>	NM_006036.4	c.881_955delinsTTTTA (p.K294Ifs8*), seq[hg19] del(2)(2p21) chr2:g.44504991_44580502del
Patient 4	POI	<i>SOX3</i>	NM_005634.2	c.1297G>T p.(Ala433Ser)
		<i>USP53</i>	NM_019050.2	c.173G>A p.(Arg58Gln), c.1253G>A p.(Arg418Gln)
		<i>UBR4</i>	NM_020765.2	c.2012G>A p.(Arg671Gln)
Patient 5	POI	<i>DNAH1</i>	NM_015512.4	c.2717A>G p.(Asp906Gly), c.4994T>C p.(Met1665Thr)
		<i>USP53</i>	NM_019050.2	c.173G>A p.(Arg58Gln), c.2975T>G p.(Phe992Cys)
		<i>ESRRG</i>	NM_001243518.1	c.55_56dupGT p.(Pro20PhefsTer36)
Patient 6	POI	<i>MRE11A</i>	NM_005591.3	c.305G>T p.(Gly102Val)
		<i>NUP107</i>	NM_020401.3	c.61C>T p.(Arg21Trp)
		<i>CAMSAP2</i>	NM_023459.2	c.1868T>C p.(Met623Thr), c.2510T>G p.(Ile837Arg)
		<i>STAG1</i>	NM_005862.2	c.634G>A p.(Asp212Asn)
Patient 7	POI	<i>MCM9</i>	NM_017696.2	c.911A>G p.(Asn304Ser)
		<i>BMPR1B</i>	NM_001203.2	c.605A>G p.(Lys202Arg)
		<i>SSH1</i>	NM_018984.3	c.151_175dup p.(Gln59LeufsTer23)
		<i>NEK8</i>	NM_178170.3	c.1795C>T p.(Arg599Ter), c.1100G>C p.(Ser367Thr)

Patient 8*	POI	<i>TP63</i>	NM_003722.4	c.1780C>T p.(Arg594Ter)
FRA125^*	POI	<i>TP63</i>	NM_003722.4	c.1794G>A p.(Trp598Ter)
Patient 9	POI	<i>CENPI</i>	NM_006733.3	c.497G>A p.(Arg166His)
Patient 10	POI (familial)	<i>NTRK3</i>	NM_002530.3	c.1495G>A p.(Asp499Asn)
Patient 11	POI	<i>REC8</i>	NM_005132.2	c.872C>T p.(Pro291Leu)
		<i>AMHR2</i>	NM_020547.3	c.1280T>G p.(Leu427Trp)
		<i>HERC1</i>	NM_003922.3	c.8236A>G p.(Thr2746Ala), c.4931T>C p.(Ile1644Thr)
		<i>RAD51C</i>	NM_058216.2	c.431T>C p.(Ile144Thr)
Patient 12	POI, polyglandular disease, Type II	<i>NUP155</i>	NM_004298.3	Chr5:37370756_37370766del (5'UTR splice site)
		<i>CCNT2</i>	NM_058241.2	c.430+1G>C Essential splice site
		<i>FOXK2</i>	NM_004514.3	c.1436C>T p.(Ala479Val), c.154C>T p.(Arg52Cys)
Patient 13	POI	<i>GREM1</i>	NM_013372.6	c.466C>T p.(Leu156Phe)
		<i>RAD50</i>	NM_005732.3	c.2177G>A p.(Arg726His), c.3907A>G p.(Ser1303Gly)

*Pathogenic variants according to ACMG guidelines, 2015 (ACMG, 2015). Refer to Supp. Table S2.

^From a different POI cohort

Further information is provided in Supp. File S3 and supplementary materials

Figures

Figure 1: Pathogenic *TP63* variants in Patient 8 and an unrelated POI patient. A) Schematic diagram depicts the *TP63* exonic structure (top) and protein isoforms (bottom). Transactivation (TA), DNA binding (DBD), oligomerization (O), sterile alpha motif (SAM) and transactivation inhibition (TI) domains are indicated. The location of the two pathogenic variants found in this study are indicated by red arrows. The location of the pathogenic variants associated with syndromic POI described in the literature are indicated by black arrows. B) Pathogenic *TP63* variants are indicated with respect to their location within the isoform relevant to oocyte function, TAp63 α . Colour depicts the phenotype of described patients. Grey: Split hand/foot malformation, purple: acro-dermato-ungual-lacrima-tooth (ADULT) syndrome, black: limb-mammary syndrome, orange: cleft palate, hypohidrosis and oligodontia, green: ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome, yellow: Rapp-Hodgkin syndrome, blue: ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome, red: isolated POI. Underlined variants have been described with POI as a feature. C) Sequence chromatogram demonstrates the *de novo* c.1780C>T p.(Arg594Ter) variant in *TP63* (NM_003722.4) in Patient 8, and the heterozygous c.1794G>A p.(Trp598Ter) variant in unrelated patient, FRA125.

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Author/s:

Tucker, EJ;Jaillard, S;Grover, SR;van den Bergen, J;Robevska, G;Bell, KM;Sadedin, S;Hanna, C;Dulon, J;Touraine, P;Sinclair, AH

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