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Sox9 downregulation in non-obstructive azoospermia by UTF1 and mediator role of POU5F1

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Abstract

Background Spermatogenesis is the process of producing mature sperm from Spermatogonial stem cells (SSCs) and this process requires a complex cooperation of different types of somatic and germ cells. Undifferentiated spermatogonia initiate the spermatogenesis and Sertoli cells as the only somatic cells inside of the seminiferous tubule play a key role in providing chemical and physical requirements for normal spermatogenesis, here, we investigated the dysfunction of these cells in non-obstructive azoospermia.

Material and method In this study, we analyzed the expression of sox9 and UTF1 in the non-obstructive human testis by immunohistochemistry. Moreover, we used the KEGG pathway and bioinformatics analysis to reveal the connection between our object genes and protein.

Results The immunohistochemistry analysis of the non-obstructive human seminiferous tubule showed low expression of Sox9 and UTF1 that was detected out of the main location of the responsible cells for these expressions. Our bioinformatics analysis clearly and strongly indicated the relation between UTF1 in undifferentiated spermatogonia and Sox9 in Sertoli cells mediated by POU5F1.

Conclusion Generally, this study showed the negative effect of POU5F1 as a mediator between Sertoli cells as the somatic cells within seminiferous tubules and undifferentiated spermatogonia as the spermatogenesis initiator germ cells in non-obstructive conditions.

Keywords Testis, Sertoli cells, Immunohistochemistry, Non-obstructive azoospermia

Introduction

In mammals, the testis is composed of complex networks of tubes that are functionally unique and are responsible for the expression of male reproductive potential [1]. Germ cells and somatic cells collaborate in testis. Functionally, germ stem cells are responsible for the production of spermatids and then sperms during spermatogenesis stages [2]. Firstly, the spermatogenesis is started by main germ cells, known as spermatogonia (Spg), which are located on the base membrane of seminiferous tubules. Spgs have two fates after the division, first is to renew main germ cells for keeping its pool as progenitor

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cells and second is to produce primary and secondary spermatocytes [3]. At the final division in the spermatogenesis, secondary spermatocytes become spermatids, which are differentiated to sperms [3].

The normal spermatogenesis needs not only normal germ cells but also an appropriate environment to provide sufficient nutrition and other chemical factors. Sertoli cells are the somatic cells of seminiferous tubules. More research on these cells proved the crucial roles of Sertoli cells in testis through providing normal spermatogenesis and thus are called nurse cells [4].

Sertoli cells have specific morphological features including irregularly-shaped cell membranes, a high number of mitochondria (indicating high metabolic activities), and large nucleus (the size of the nucleus in Sertoli cells depends on the developmental age and the stages of spermatogenesis) [5]. Generally, Sertoli cells mainly take part in the formation of the blood-testis barrier (BTB), the production and differentiation of germ cells, phagocytosis activities, the degeneration of the abnormal sexual cells, the production and secretion of regulatory hormones, the creation of a safe environment for spermatogenesis, and the expression of hormonal receptors in reproductive processes [6–8].

Utf1 is a pluripotency-associated gene and was found to be expressed in embryonic stem (ES) cells, embryonic carcinoma (EC) cells, and primordial germ cells (PGC). Several investigations indicated the UTF1 role in the proliferation stage of ES cells. Furthermore, it was suggested that activation of UTF1 is an important mechanism by which POU5F1 maintains the stem cell state of ES cells [9].

Sox9 is Sry-box containing gene that encodes a transcriptional activator. Sox9 expression is restricted to the Sertoli cell lineage and persists in the adult [10]. Sox9 gene has two independent activity amplifying pathways [8, 11]. First is production of testicular L-PGDS (Lipocalin-Type Prostaglandin D Synthase) leads to the accumulation of PGD2 (Prostaglandin D2), which in turn activates Sox9 transcription and nuclear translocation of SOX9 [12, 13]. This mechanism participates together with FGF9 (fibroblast growth factor 9) as an amplification system of Sox9 gene expression and activity during mammalian testicular organogenesis [14–17].

The main aim of the present investigation was understanding of correlation gene expression pattern between germ cells and somatic cells in non-obstructive azoospermia. Our results would be helpful for further researches with a deep focus on finding treatment for infertility like non-obstructive azoospermia.

Materials and methods

In this experimental study, we used human non-obstructive samples from the 2 adults male patient's biopsies. They were obtained from Institute of Anatomy and Cell biology, Medical Faculty, University of Heidelberg. Also, testis samples from 3 pair's mice (C57BL/6 strain) were obtained from pasture institute and after putting in an enzymatic digestive solution including Dispase (0.5 mg / ml) (Sigma Aldrich), DNAse (0.5 mg / ml) (Sigma Aldrich), and Collagenase (0.5 mg / ml) (Sigma Aldrich) used for further Immunostaining process.

Immunohistochemical staining

Testis samples were fixed in 4% paraformaldehyde. After cutting of the testis tissue blocks with a microtome (about 10 µm thickness), sections were mounted on slides and stored at room temperature (RT) until used. Before staining, all of the segments were deparaffinized with xylene and rehydrated in an ethanol arrangement. After heat mediated antigen retrieval (10 mM Sodium Citrate Buffer, pH 6 or 1 mM EDTA, pH 8), non-specific binding was blocked with 10% serum and 0.3% Triton in PBS and immunofluorescence staining was performed as explained above (at 95 °C for 20 min).

PPI network construction and analysis

To construct the PPI network, we used the Search Tool for the STRING version 11.5 (Retrieval of Interacting Genes/Proteins database, https://string-db.org/). STRING is a web database intends to integrate all known and predicted interactions between proteins, including physical interactions and functional associations (Szklarczyk, Gable et al. 2021). STRING app in Cytoscape Software (v 3.8.2) was used to construct the PPI network.

Gene enrichment analysis

To investigate the functions of the validated genes involved in the sub-network, we have performed the STRING Enrichment analysis in the Cytoscape Software.

Results

As a control experiment, we used Sox9 and UTF1 expression in mice seminiferous tubules by Immunohistochemistry analysis (Fig. 1). Our analysis revealed the expression of both markers near the basal membrane as we expected based on the specific expression of Sox9 in Sertoli cells (Fig. 1A) and UTF1 in undifferentiated germ cells (Fig. 1B). Confocal scanning UV-laser microscope images proved the expression of UTF1 and sox9 out of the common compartment of seminiferous tubules of non-obstructive azoospermia (Fig. 2). The giant component of the "Stem cell" PPI network generated by STRING and Cytoscape consisted of 100 nodes and 1996 edges (All nodes represent proteins and edges

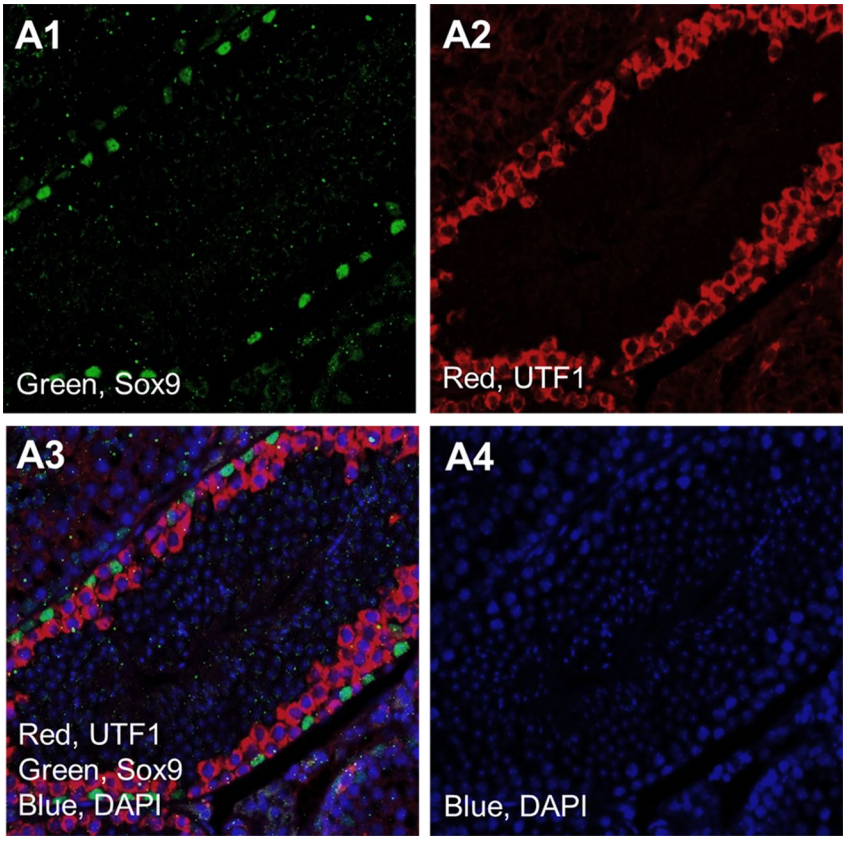


Fig. 1 Immunohistochemistry analysis in mice seminiferous tubule as a control pattern. Sox9 expression in the Sertoli cells (**A1**), UTF1 expression (**A2**), Merge image of UTF1 and Sox9 and blue DAPI (**A3**). Blue DAPI for nuclear staining (**A4**). (Scale bar = 50 µm)

represent protein-protein associations). According to the network, UTF1, Sox9 and POU5F1 are three nodes that we set them first (Fig. 3). We further searched the first neighbors' nodes with these three genes and we generate a sub-network. This sub-network includes 37 nodes. This suggests that UTF1, Sox9 and POU5F1 are in a highly tight correlation with other stem cell genes. We implemented STRING Enrichment analysis on 14 genes that we had selected, to evaluate molecular functions and cellular locations associated with UTF1, Sox9 and POU5F1 (Fig. 3). We selected some biological processes based on the aim of our experimental studies including; embryo development, stem cell differentiation, and stem cell population maintenance. Also, by TISSUES analysis the localization of genes in pluripotent stem cells, trophectoderm, spermatogonium, adult stem cell, embryoid body, ectoderm, blastocyst, morula, and embryonic stem cell. Furthermore, by KEGG Pathways we identified that if our genes participate in the signaling pathways regulating pluripotency of stem cells. In addition, with COMPARTMENTS analysis we show the localization of our genes in cells (Fig. 4).

Discussion

In the testicular niche, supportive Sertoli cells and interstitial tissue cells produce various growth factors that stimulate the self-renewal, proliferation, and differentiation of SSCs. Male infertility can arise from factors such as hormonal imbalances, physical challenges, psychological or behavioral issues [18]. Physical problems in the testis, such as a low number or absence of SSCs or supportive cells, can contribute to male infertility [19]. Currently, patients with deficiencies in both germ cells and somatic cells (supportive cells) in the testis face challenges, leading to the differentiation of undifferentiated SSCs into sperm.

Sertoli cells play a crucial role in regulating spermatogonial cell functions during spermatogenesis in the male testes [20]. The Sox gene family, particularly Sox9, serves as a transcription factor critical for cellular differentiation during embryogenesis in various tissues, including Sertoli cells of the testis [21], neural crest cells, and chondrocytes [22, 23]. Since Sox9 expression is limited to Sertoli cells and is used as a specific marker for their identification in the testis, we utilized Sox9 as a Sertoli-specific marker in this study [24, 25]. It is known that Sox9 also plays a crucial role in vertebrate sex determination, regulated by the SRY gene [22]. Studies have shown

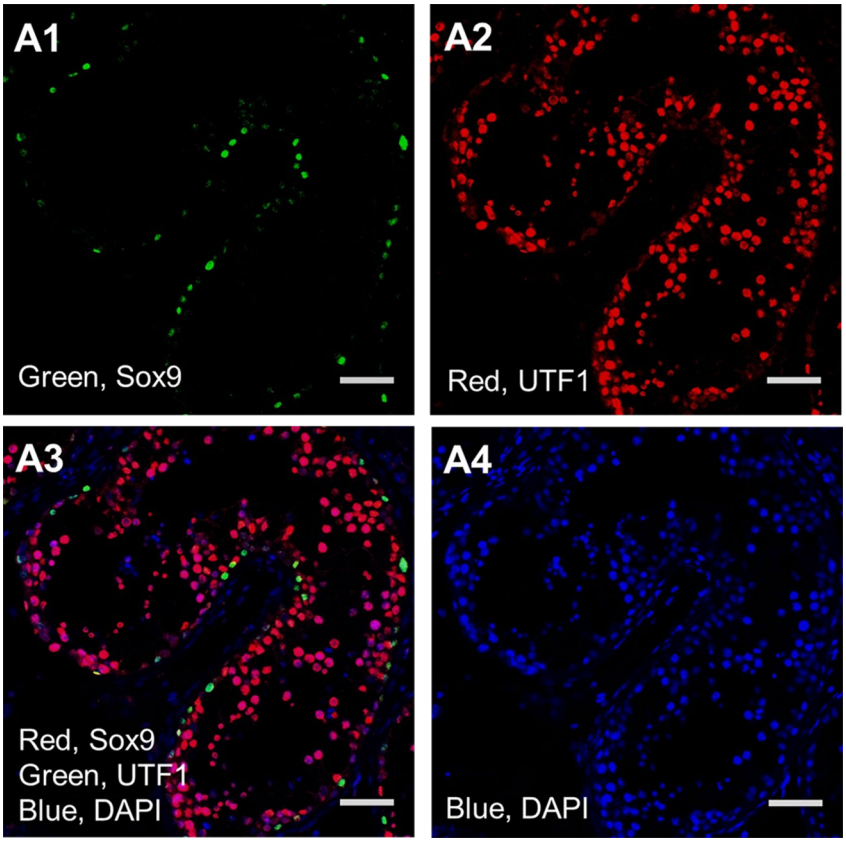
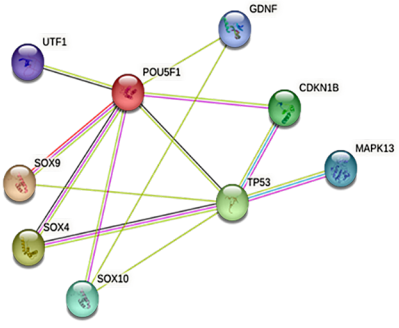


Fig. 2 Immunohistochemistry analysis of non-obstructive human seminiferous tubule. Sox9 expression in the Sertoli cells (**A1**), UTF1 expression (**A2**), Merge image of UTF1 and Sox9 and blue DAPI (**A3**). Blue DAPI for nuclear staining (**A4**). (Scale bar = 50 µm)



POU5F1	POU domain, class 5, transcription factor 1; Transcription factor that binds to the octamer motif (5'-ATTTGCAT-3'). Forms a trimeric complex with SOX2 on DNA and controls the expression of a number of genes involved in embryonic development such as YES1, FGF4, UTF1 and ZFP206. Critical for early embryogenesis and for embryonic stem cell pluripotency (360 aa)
SOX9	Transcription factor SOX-9; Transcriptional regulator. Binds to the COL2A1 promoter and activates COL2A1 expression, as part of a complex with ZNF219 (By similarity). Plays a role in chondrocyte differentiation (By similarity). Important for normal skeletal development; SRY-boxes (509 aa)
SOX4	Transcription factor SOX-4; Transcriptional activator that binds with high affinity to the T-cell enhancer motif 5'-AACAAAG-3' motif; SRY-boxes (474 aa)
TP53	Cellular tumor antigen p53; Acts as a tumor suppressor in many tumor types; induces growth arrest or apoptosis depending on the physiological circumstances and cell type. Involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process. One of the activated genes is an inhibitor of cyclin-dependent kinases. Apoptosis induction seems to be mediated either by stimulation of BAX and FAS antigen expression, or by repression of Bcl-2 expression. In cooperation with mitochondrial PPIF is involved in [...] (393 aa)
CDKN1B	Cyclin-dependent kinase inhibitor 1B; Important regulator of cell cycle progression. Inhibits the kinase activity of CDK2 bound to cyclin A, but has little inhibitory activity on CDK2 bound to SPDYA. Involved in G1 arrest. Potent inhibitor of cyclin E- and cyclin A- CDK2 complexes. Forms a complex with cyclin type D-CDK4 complexes and is involved in the assembly, stability, and modulation of CCND1-CDK4 complex activation. Acts either as an inhibitor or an activator of cyclin type D-CDK4 complexes depending on its phosphorylation state and/or stoichometry; Belongs to the CDI family (198 aa)
SOX10	Transcription factor SOX-10; Transcription factor that plays a central role in developing and mature glia. Specifically activates expression of myelin genes, during oligodendrocyte (OL) maturation, such as DUSP15 and MYRF, thereby playing a central role in oligodendrocyte maturation and CNS myelination. Once induced, MYRF cooperates with SOX10 to implement the myelination program. Transcriptional activator of MITF, acting synergistically with PAX3; SRY-boxes (466 aa)
MAPK13	Mitogen-activated protein kinase 13; Serine/threonine kinase which acts as an essential component of the MAP kinase signal transduction pathway. MAPK13 is one of the four p38 MAPKs which play an important role in the cascades of cellular responses evoked by extracellular stimuli such as proinflammatory cytokines or physical stress leading to direct activation of transcription factors such as ELK1 and ATF2. Accordingly, p38 MAPKs phosphorylate a broad range of proteins and it has been estimated that they may have approximately 200 to 300 substrates each. MAPK13 is one of the less studie [...] (365 aa)
GDNF	Glial cell line-derived neurotrophic factor; Neurotrophic factor that enhances survival and morphological differentiation of dopaminergic neurons and increases their high-affinity dopamine uptake; Belongs to the TGF-beta family. GDNF subfamily (228 aa)
UTF1	Undifferentiated embryonic cell transcription factor 1; Acts as a transcriptional coactivator of ATF2 (341 aa)

Fig. 3 The functional enrichment analysis of selected genes that have direct interaction with UTF1 and Sox9. Different colored parts of the circles refer to the related biological processes and with line thickness being indicative of evidence strength for a predicted interaction

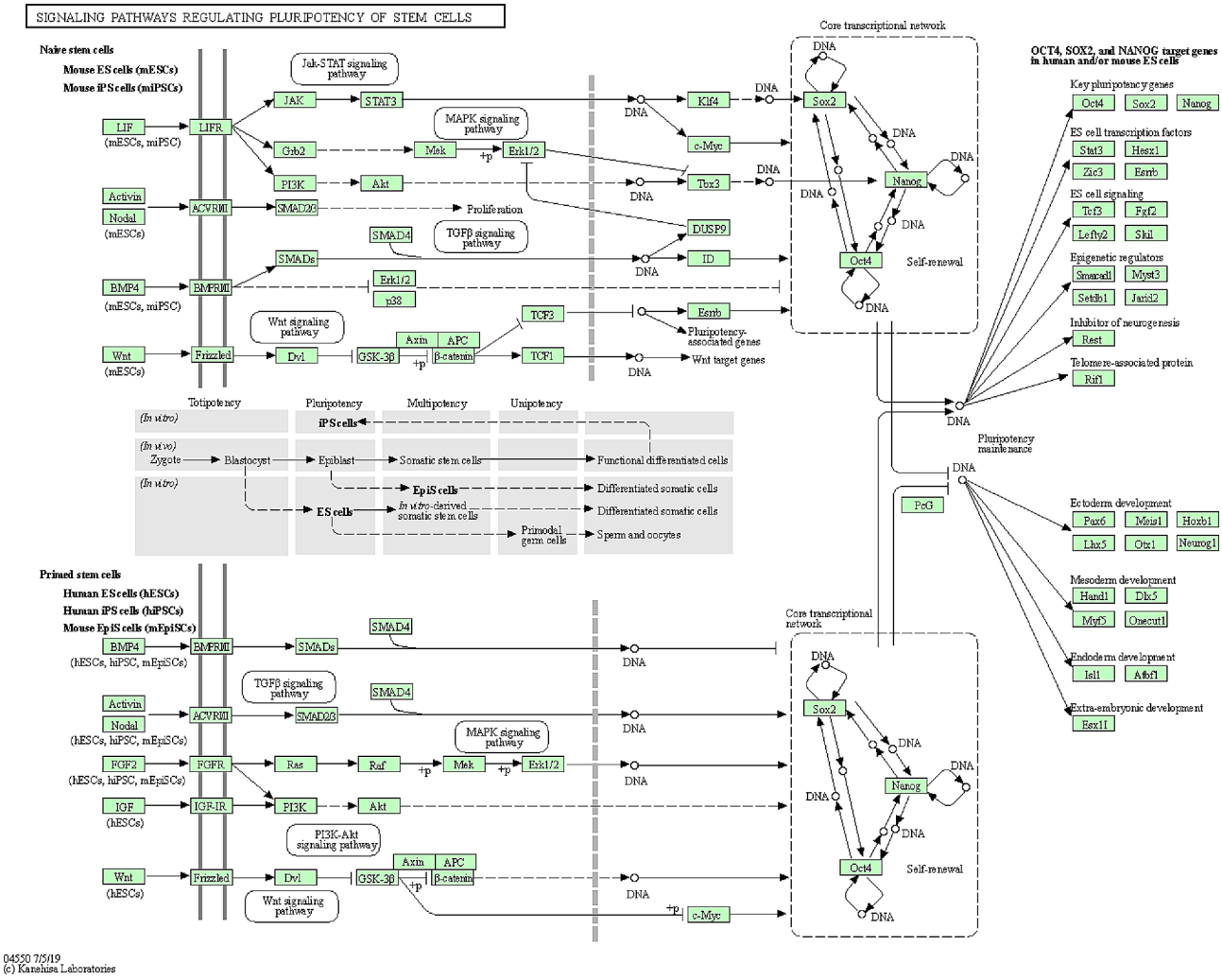


Fig. 4 KEGG view on Signaling pathways regulating pluripotency of stem cells

that the pattern of Sox9 expression in Sertoli cells differs significantly in patients with non-obstructive azoospermia or Sertoli cells only syndrome (SCOS). Kuo-Chung Lan et al. reported up-regulation of Sox9 protein expression and a different expression pattern in patients with SCOS [23]. Given the critical roles of Sertoli cells in male reproduction determination, several investigations have focused on the chemical and physiological features of Sertoli cells to understand their exact mechanisms in supporting spermatogenesis [26–31].

On the other hand, UTF1 is a specific marker for the undifferentiated compartment and was utilized in the human non-obstructive azoospermia testis in this study. Immunohistochemistry results indicated a reduction in Sox9 and UTF1 expression in human non-obstructive testes, clearly outside the main Sertoli and undifferentiated cells' niche. Our bioinformatics analysis and signaling pathway investigation revealed the impact of POU5F1 on UTF1 expression in undifferentiated spermatogonia and demonstrated a significant connection with Sox9 expression. This might explain the reduction of these genes and proteins in non-obstructive azoospermia testes.

Conclusion

In this project we confirmed that there is correlation between the POU5F1 and regulation of the Sox9 (Sertoli-specific marker) and UTF1 (undifferentiated germ cell marker). Our results from immunohistochemistry of non-obstructive azoospermia revealed a low expression of sox9 and UTF1 in the locations that were not related to the Sertoli cells and undifferentiated spermatogonia respectively. Our bioinformatics analysis indicated a mediatory role of POU5F1 between UTF1 and Sox9. The presented informations will be useful for further advanced studies against infertility.

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Author contributions
M.M.R.; Wrote the manuscript, data analysis and edited the final document. H.A.; carried out and design the experiment, assembly of data and data analysis; A.N. and M.A.M.; Wrote the manuscript, data analysis. The authors read and approved the final manuscript.

Funding
Not applicable.

Data availability
The data sets analyzed for the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Informed consent, was obtained from all participants by Heidelberg university that we obtained the samples and all stages of experiments approved by the ethical committee of Amol University of Special Modern Technologies (Ir.ausmt.rec.1400.03). All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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