Universidad de Oviedo

UNIVERSIDAD DE OVIEDO

PROGRAMA DE DOCTORADO
CIENCIAS DE LA SALUD

Órganos de los sentidos y Sistema Nervioso Periférico

Tesis Doctoral

Mecanosensibilidad mediada por PIEZO2

Yolanda García Mesa

Directores
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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español: **MECANOSENSIBILIDAD MEDIADA POR PIEZO2**	Inglés: **MECHANOSENSIBILITY MEDIATED BY PIEZO2**

2.- Autor	
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RESUMEN (en español)

Introducción. La mecanobiología es el efecto causado por la acción de la fuerza sobre la biología de las células, con independencia de su filiación histológica. En los órganos sensitivos cutáneos complejos (OSCC), en la periferia de los mecanorreceptores de bajo umbral (MRBU), se originan los estímulos nerviosos mecánicos en virtud de un proceso denominado mecanotransducción, que consiste en la conversión de estímulos mecánicos en señales eléctricas; en este contexto, el sentido del tacto es un excelente ejemplo de mecanobiología en el sistema nervioso periférico. Cada morfotipo de OSCC se supone que detecta diferentes cualidades de somatosensibilidad mecánica. Los mecanismos moleculares de la mecanotransducción en los MRBU cutáneos (de adaptación rápida y lenta) se sustentan en la expresión de canales iónicos mecanosensibles, especialmente de la familia Piezo (PIEZO1 y PIEZO2). Por otro lado, existen evidencias sobre la expresión y funcionalidad de las proteínas PIEZO en tejidos no nerviosos, y estudios recientes relacionan la expresión de las proteínas PIEZO con diferentes patologías humanas, especialmente con el cáncer.

Hipótesis y objetivos.- **Hipótesis 1:** PIEZO2 es un canal activado mecánicamente requerido para la discriminación táctil y debe expresarse en alguno de los tipos celulares de los órganos sensitivos cutáneos complejos (OSCC). **Hipótesis 2:** PIEZO2 es el responsable de la mecanotransducción en todos los sistemas biológicos mecanosensibles. **Hipótesis 3:** La expresión de tisular de PIEZO2 está alterada en patologías que cursan con alteraciones de la sensibilidad, mecánica o no, y es positivo en los tumores derivados de células que expresan PIEZO2.

Objetivos: 1) Estudiar la presencia y localización del canal PIEZO2 en los OSCC de la piel glabra humana (piel digital, del prepucio y del clítoris). 2) Establecer el momento del desarrollo embrionario en el OSCC de la piel glabra de los dedos comienzan a expresar PIEZO2 como indicador de la adquisición de competencia, 3) Identificar las células mecanosensibles en el sistema genito-urinario humano en base a la expresión de PIEZO2; 4) Analizar los cambios en la expresión de PIEZO2 en los OSCC en el curso de la neuropatía diabética; 5) Estudiar si los tumores derivados de células PIEZO2 positivas mantienen o alteran los patrones de expresión durante la transformación tumoral (merkeloma).

Material y técnicas, Resultados, y Discusión.- La tesis está conformada por un compendio de publicaciones que contienen de manera detallada y reproducible el Material y Técnicas, así como los resultados y las discusiones específicas. Los artículos incluidos, por orden cronológico de publicación son:

1.- **García-Mesa Y**, et al. Merkel cells and Meissner's corpuscles in human digital skin display Piezo2 immunoreactivity. **J Anat**. 2017; 231: 978-989. doi: 10.1111/joa.12688.
2.- **García-Mesa Y**, et al. Sensory innervation of the human male prepuce: Meissner's corpuscles predominate. **J Anat**. 2021; 239:892-902. doi: 10.1111/joa.13481.
3.- **García-Mesa Y**, et al. Glans clitoris innervation: PIEZO2 and sexual mechanosensitivity. **J Anat**. 2021; 238:446-454. doi: 10.1111/joa.13317.
4.- **García-Mesa Y**, et al. Involvement of Cutaneous Sensory Corpuscles in Non-Painful and Painful Diabetic Neuropathy. **J Clin Med**. 2021; 10:4609. doi: 10.3390/jcm10194609.

5.- **García-Mesa** Y, et al. The acquisition of mechanoreceptive competence by human digital Merkel cells and sensory corpuscles during development: An immunohistochemical study of PIEZO2. **Ann Anat**. 2022; 243:151953. doi: 10.1016/j.aanat.2022.151953.
6.- **García-Mesa Y**, et al. **Ann Anat**. 2022; 243:151955. doi: 10.1016/j.aanat.2022.151955.
7.- **García-Mesa Y**, et al. Merkel cell carcinoma display and PIEZO2 immunoreactivity. **J Personalized Med**. 2022; 12:894. doi: 10.3390/jpm12060894.
8.- **García-Mesa Y**, et al. Immunohistochemical localization of PIEZO1 and PIEZO2 in human male and female urinary and genital apparatus. Int J Mol Sci., sometido.

Conclusiones.- 1) En los órganos sensitivos cutáneos complejos considerados como mecanorreceptores de bajo umbral y adaptación rápida (corpúsculos de Meissner y relacionados y corpúsculos de Pacini) el axón expresa PIEZO2 con independencia de su localización anatómica (piel digital, del clítoris y del glande). Por el contrario, en los de adaptación lenta (complejos célula de Merkel-neurita) los axones son PIEZO2 negativos mientras que las células accesorias PIEZO2 positivas. 2) En base a la expresión de PIEZO2 las células de Merkel de la piel digital humana adquieren mecanocompetencia a las semanas 22-23 semanas de edad de gestación estimada, y los corpúsculos de Meissner y Pacini a partir de las 11 semanas de edad de gestación estimada. 3) En el riñón y vías urinarias humanas, así como en los diferentes órganos de los aparatos genitales femenino y masculino, se detectaron poblaciones de células epiteliales PIEZO2 (y PIEZO1) positivas. 4) La neuropatía diabética dolorosa cursa con un deterioro progresivo de la estructura de los corpúsculos sensitivos de la pile glabra humano, consistente con denervación, acompañado de la expresión de proteínas mecanosensibles. 6) Las células del carcinoma de células de Merkel con PIEZO2 positivas.

RESUMEN (en inglés)

Introduction. Mechanobiology is the effect caused by the action of force on the biology of cells, regardless of their histological affiliation. In complex cutaneous sensory organs (CCSO) on the periphery of low-threshold mechanoreceptors (LTMRs), mechanical nerve stimuli originate by virtue of a process called mechanotransduction that consists of the conversion of mechanical stimuli into electrical signals; In this context, the sense of touch is an excellent example of mechanobiology in the peripheral nervous system. Each CCSO morphotype is supposed to detect different qualities of mechanical somatosensitivity. The molecular mechanisms of mechanotransduction in cutaneous LTMRs (fast and slow adapting) are based on the expression of mechanosensitive ion channels, especially of the Piezo family (PIEZO1 and PIEZO2). On the other hand, there is evidence of expression and functionality of PIEZO proteins in non-nervous tissues, and recent studies relate the expression of PIEZO proteins with different human pathologies, especially cancer.

Hypothesis and objectives. - **Hypothesis 1:** PIEZO2 is a mechanically gated channel required for tactile discrimination and must be expressed in one of the cell types of complex cutaneous sensory organs (CCSO). **Hypothesis 2:** PIEZO2 is responsible for mechanotransduction in all mechanosensitive biological systems. **Hypothesis 3:** The tissue expression of PIEZO2 is altered in pathologies that course with alterations of sensitivity, mechanical or not, and is positive in tumors derived from cells that express PIEZO2.
Objectives: 1) Study the occurrence and location of PIEZO2 in the CCSO of human glabrous skin (digital, foreskin and clitoris skin). 2) Establish the time of embryonic development in the CCSO of the digital glabrous skin begin to express PIEZO2 as an indicator of the acquisition of competence, 3) Identify the mechanosensitive cells in the human genito-urinary system based on the expression of PIEZO2; 4) Analyze the changes in PIEZO2 expression in CCSO in the course of diabetic neuropathy; 5) Study whether tumors derived from PIEZO2 positive cells maintain or alter expression patterns during tumor transformation (merkeloma).

Material and techniques, Results, and **Description**.- The thesis is made up of a compendium of scientific articles that contain in a detailed and reproducible way the Material and Techniques, as well as the Results and specific Discussion. The articles included, in chronological order of publication are:
1.- **García-Mesa Y**, et al. Merkel cells and Meissner's corpuscles in human digital skin display

Piezo2 immunoreactivity. **J Anat**. 2017; 231: 978-989. doi: 10.1111/joa.12688.
2.- **García-Mesa Y**, et al. Sensory innervation of the human male prepuce: Meissner's corpuscles predominate. **J Anat**. 2021; 239:892-902. doi: 10.1111/joa.13481.
3.- **García-Mesa Y**, et al. Glans clitoris innervation: PIEZO2 and sexual mechanosensitivity. **J Anat**. 2021; 238:446-454. doi: 10.1111/joa.13317.
4.- **García-Mesa Y**, et al. Involvement of Cutaneous Sensory Corpuscles in Non-Painful and Painful Diabetic Neuropathy. **J Clin Med**. 2021; 10:4609. doi: 10.3390/jcm10194609.
5.- **García-Mesa** Y, et al. The acquisition of mechanoreceptive competence by human digital Merkel cells and sensory corpuscles during development: An immunohistochemical study of PIEZO2. **Ann Anat**. 2022; 243:151953. doi: 10.1016/j.aanat.2022.151953.
6.- **García-Mesa Y**, et al. **Ann Anat**. 2022; 243:151955. doi: 10.1016/j.aanat.2022.151955.
7.- **García-Mesa Y**, et al. Merkel cell carcinoma display and PIEZO2 immunoreactivity. **J Personalized Med**. 2022; 12:894. doi: 10.3390/jpm12060894.
8.- **García-Mesa Y**, et al. Immunohistochemical localization of PIEZO1 and PIEZO2 in human male and female urinary and genital apparatus. Int J Mol Sci., submit.

Concluding remarks. - 1) In the complex cutaneous sensory organs regarded as and rapidly adapted low threshold (Meissner and related corpuscles, and Pacinian corpuscles) the axon expresses PIEZO2 regardless of its anatomical location (digital, clitoris and glans skin). By contrast, in slowly-adapting axons (Merkel cell-neurite complexes) axons are PIEZO2 negative while accessory cells are PIEZO2 positive. 2) Based on the expression of PIEZO2 Merkel cells of human digital skin acquire mechanocompetence at 22-23 weeks of estimated gestation age, and Meissner and Pacini corpuscles from 11 weeks of estimated gestation age. 3) In the kidney and human urinary tract, as well as in the different organs of the female and male genital tracts, PIEZO2 (and PIEZO1) positive epithelial cell populations were detected. 4) Painful diabetic neuropathy courses with a progressive deterioration of the structure of the sensory corpuscles of the human gabrous skin, consistent with denervation, accompanied by loss of expression of mechanosensitive proteins. 6) Merkel cell carcinoma cells are PIEZO2 positive.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN CIENCIAS DE LA SALUD

DEDICATORIA

A mi pilar durante todo este camino, gracias (A).

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A mis directores de tesis….
A mi familia….
A mis amigos….
A mis compañeros de trabajo….

ÍNDICE

1. Introducción

1. Introducción

La ***mecanobiología*** puede definirse como el efecto causado por la acción de la fuerza sobre la biología de las células. En ella se incluyen efectos tan dispares como la mecanosensación, y el tacto en particular, o la sensación de saciedad que desencadena la distensión de las paredes del tubo gastrointestinal.

La sensación táctil es uno de los componentes de la mecanosensación y se origina en fibras nerviosas especiales que se diferencian por su estructura (son axones gruesos mielinizados), fisiología (la velocidad de conducción de sus potenciales de acción) y forma de terminar en la piel (terminaciones nerviosas libres y corpúsculos sensitivos). Estas formaciones nerviosas sensitivas cutáneas (también denominadas corpúsculos sensitivos u órganos sensitivos cutáneos complejos) son los receptores para muchas modalidades de mecanosensibiliad como tacto, sensación de presión, estiramiento y vibración (McGlone y Reilly, 2010; Zimmerman et al., 2014; Cobo et al., 2021; Handler y Gintly, 2021; Martín-Alguacil et al., 2021; Vega y Suazo, 2021).

Las fibras nerviosas mecanosensitivas son, principalmente, las fibras Aβ, pero ocasionalmente pueden funcionar como tales, fibras Aδ y fibras C, y dependen de mecanorreceptores de bajo umbral (*low-threshold mechanoreceptors*: LTMRs). Las neuronas LTMR son pseudomonopolares y la prolongación periférica de su axón se distribuye por la piel donde se asocian con células especializadas: células de Merkel (formando complejos de células de Merkel-axón), células Schwann-like que forman parte de los corpúsculos sensitivos (corpúsculos de Meissner, corpúsculos de Pacini y corpúsculos de Ruffini) o células de folículos pilosos (terminaciones nerviosas sensitivas asociadas a folículos pilosos) (**Figura 1**) (Rice y Albrecht, 2008; Li et al., 2011; Abraira y Ginty, 2013; Djouhri, 2016a,b; Martín-Alguacil et al., 2021; Vega y Suazo, 2021; Suazo et al., 2022).

En los órganos sensitivos cutáneos complejos (OSCC), es decir, en la periferia de los LTMR, se originan los estímulos nerviosos mecánicos en virtud de un proceso denominado ***mecanotransducción***, que consiste en la conversión de estímulos mecánicos en señales

eléctricas (Li et al., 2011; Roudaut et al., 2012; Fleming y Luo, 2013; Cobo et al., 2020); en este contexto, el sentido del tacto es un excelente ejemplo de mecanobiología en el sistema nervioso periférico (Chalfie, 2009; Hoffman et al., 2011; Schneider et al., 2016). Cada morfotipo de OSCC se supone que detecta diferentes cualidades de somatosensibilidad mecánica (ver Martín-Alguacil et al., 2021). Por lo tanto, entender la mecanotransducción en las terminaciones de los LTMRs cutáneos requiere la identificación de los mecanismos moleculares que transducen la deformación de las células que los forman en potenciales de acción específicos en el LTMR correspondiente (ver Suazo et al., 2022).

En la piel glabra se han identificado cuatro tipos de LTMR, dos de adaptación lenta (*slowly adapting* LTMRs: SA-LTMTs) y otros dos de adaptación rápida (*rapidly adapting* LTMR: RA-LTMRs) y, a su vez, dos tipos dentro de cada uno de ellos: SA-LTMR de tipo I que se corresponden con los complejos axón-célula de Merkel; SA-LTMRs de tipo II representados por los corpúsculos de Ruffini; RA-LTMRs de tipo I que son los corpúsculos de Meissner; y RA-LTMRs de tipo II correspondientes a los corpúsculos de Pacini (Albrecht, 2008; Fleming y Luo 2013; Zimmerman et al., 2014). Los SA-LTMR de tipo I están involucrados en el tacto fino, los SA-LTMR de tipo II en la detección del estiramiento y los RA-LTMR están sintonizados con la vibración y el desplazamiento a través de la piel (Johnson, 2001; Jones y Smith, 2014; Owens y Lumpkin, 2014; Olson et al., 2016). Lo que llama poderosamente la atención es la existencia de tantos tipos de receptores para el desarrollo de una función tan aparentemente simple como el tacto (Vega y Suazo, 2021).

Clásicamente, se ha venido considerando que las propiedades mecánicas de las células periaxionales que forman corpúsculos sensitivos, así como las diferenciaciones en la membrana axónica eran necesarias y suficientes para generar potenciales de acción. Sin embargo, el descubrimiento de que algunos canales iónicos están en la base de la sensibilidad y que factores mecánicos pueden activarlos (canales de iones mecanosensitivos o "cerrados/abiertos" mecánicamente), ha llevado a concluir que la mecanotransducción se produce a través de ellos. En confirmación de esta teoría está el hecho de que los LTMR que inervan la piel (Lumpkin y Caterina, 2007; Tsunozaki y

Bautista, 2009; Lumpkin et al., 2010; Gu y Gu, 2014; Paluch et al., 2015; Ranade et al., 2015; Wu et al., 2017) y sus células diana cutáneas (Cobo et al., 2020a) expresan canales iónicos activados por la fuerza o el desplazamiento. En otras palabras, la apertura/cierre de canales iónicos presentes en los mecanorreceptores cutáneos en respuesta a estímulos mecánicos es el primer paso para transducir energía mecánica en la actividad eléctrica (Delmas y Coste, 2013; Ranade et al., 2015).

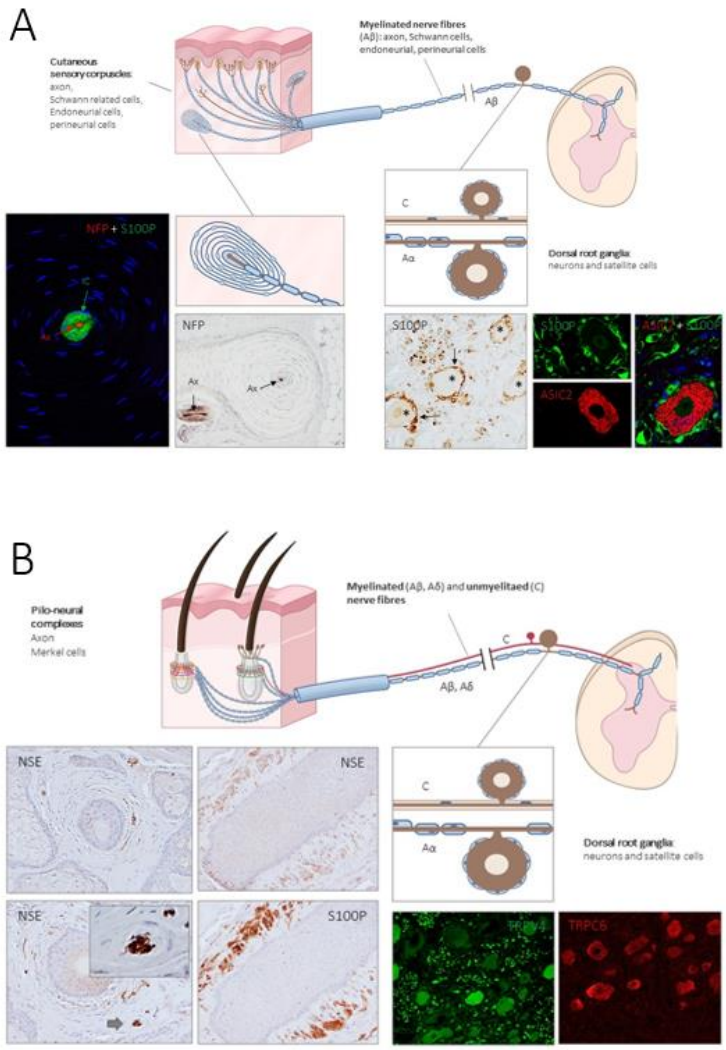

Figura 1. Representación esquemática y fotografías de A la inervación glabra (complejos pilo-neurales y ganglios raquídeos) y B de la piel con pelo (complejos pilo-neurales y ganglios sensitivos) humana. Tomada de Cobo et al. (2021)

Según Delmas y Coste (2013), los canales iónicos mecanosensibles se pueden dividir en dos categorías: los que responden a la tensión de la membrana y los que son susceptibles a estiramientos. La evidencia experimental sugiere que existen al menos tres mecanismos capaces de activar los canales iónicos que se cierran/abren mecánicamente: 1) modificaciones de la membrana celular en las proximidades de los canales; 2) tensión de la matriz extracelular y/o proteínas del citoesqueleto ancladas a los dominios extra o intracitoplasmáticos, respectivamente, de los canales iónicos de membrana; y, 3) acoplamiento con proteínas mecanosensibles secundarias a los canales iónicos (Lumpkin y Caterina, 2007; Sharif-Naeini, 2015). Por lo tanto, cualquiera de estos tres mecanismos, o una combinación de ellos, puede determinar la apertura/cierre de canales iónicos mecanosensibles y, en consecuencia, ser responsable de la mecanotransducción en los LTMRs.

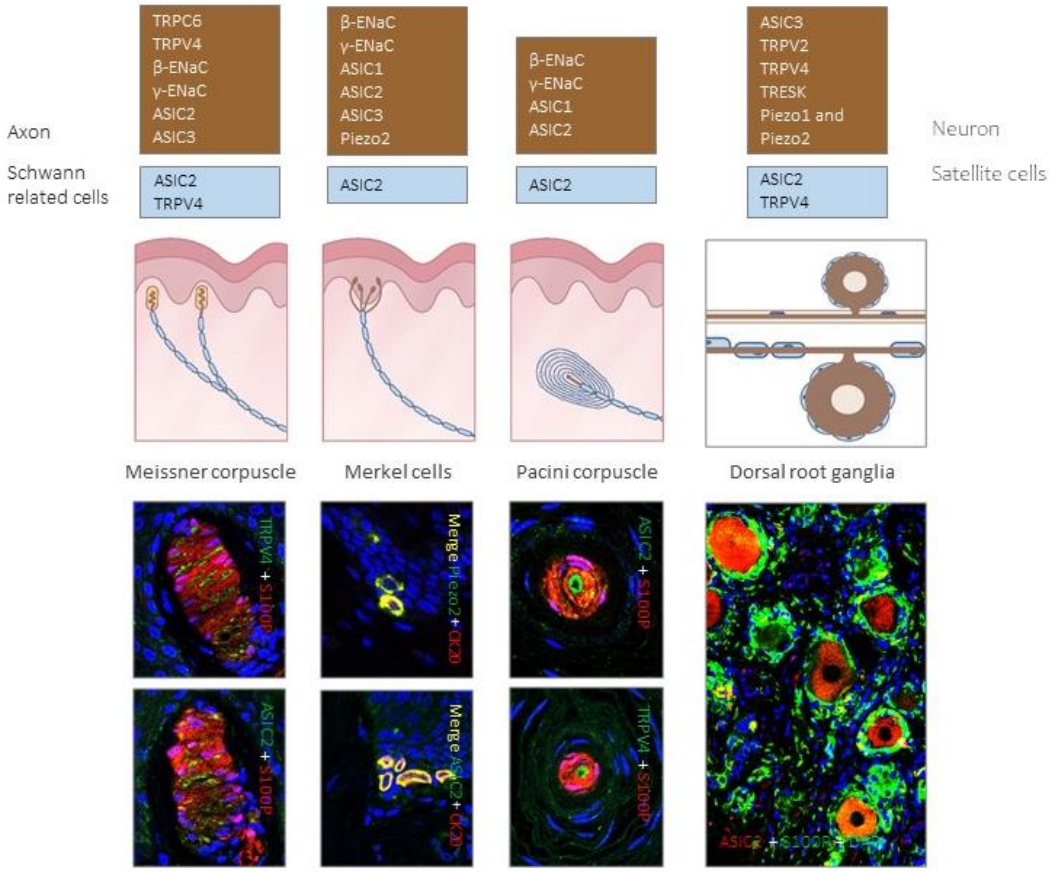

Figura 2. Expresión de diferentes canales iónicos mecanosensibles en los LTMRs, tanto a nivel de los corpúsculos sensitivos como las neuronas de los ganglios raquídeos. Tomada de Cobo et al. (2020)

La participación de los canales iónicos en la mecanosensibilidad es universalmente aceptada, aunque los canales por sí mismos no pueden explicar el complejo proceso de mecanotransducción. De hecho, algunos experimentos han demostrado que hay algo más que canales iónicos en la génesis de los potenciales de acción, y que la especificidad de la respuesta mecanosensitiva está relacionada con la arquitectura celular (Lin et al., 2009; Sachs, 2010). En la actualidad se sabe que varios canales iónicos de las familias degenerina/canales epiteliales de sodio (DEG/ENa^{+}C), receptor de potencial transitorio (TRP), dominio de dos poros de potasio (K_{2p}) y Piezo, han demostrado su potencial como canales de iones mecanotransductores total o parcialmente (Gillespie y Walker, 2001; Lumpkin y Caterina, 2007; Delmas y Coste, 2013; Ranade et al., 2015). Todos ellos están presentes en los LTMRs de los vertebrados (ver para una revisión Cobo et al., 2020b).

Sin embargo, los fenotipos sensoriales de ratones deficientes para estas proteínas no siempre apoyan un papel clave en la mecanotransducción, y sólo PIEZO2 ha demostrado propiedades mecanotransductoras completas en vertebrados (Delmas y Coste, 2013; Ranade et al., 2015; Wu et al., 2017). Por lo tanto, el resto de los canales iónicos considerados como mecanosensibles podrían ser accesorios y no una parte esencial en el proceso.

El presente trabajo de Tesis Doctoral se centra en una de las proteínas de la familia Piezo, PIEZO2, y se presenta como recopilación de trabajos. Todo el estudio se ha realizado sobre muestras de material humano.

En primer lugar, se incluye un estudio sobre la distribución de la sinaptofisina en el sistema nervioso periférico que ha demostrado la utilidad de esta proteína de las vesículas de sinapsis como marcador axónico periférico. Este trabajo se realizó por la necesidad de detectar marcadores axónicos específicos que pudieran servir en los experimentos de doble inmunomarcaje (*Synaptophysin is a selective marker for axons in human cutaneous end organ complexes*. Ann Anat. 2022; 243:151955).

A continuación, se incluye un primer bloque de trabajos encaminado a estudiar la distribución de la proteína PIEZO2 en los corpúsculos sensitivos de la piel glabra humana. Consta de cuatro trabajos de investigación que analizan la presencia de PIEZO2 en la piel digital (*Merkel cells and Meissner's corpuscles in human digital skin display Piezo2 immunoreactivity*. J Anat. 2017; 231: 978-989), el clitoris (*Glans clitoris innervation: PIEZO2 and sexual mechanosensitivity*. J Anat. 2021; 238:446-454) y el prepucio (*Sensory innervation of the human male prepuce: Meissner's corpuscles predominate*. J Anat. 2021; 239:892-902). Se incluye, también, un estudio encaminado a establecer cuándo se adquiere la capacidad de mecanotransducción de los LTMRs de la piel glabra humana durante el desarrollo (*The acquisition of mechanoreceptive competence by human digital Merkel cells and sensory corpuscles during development: An immunohistochemical study of PIEZO2*. Ann Anat. 2022; 243:151953).

Algunos trabajos recientes relacionan la expresión de las proteínas PIEZO con diferentes patologías humanas, especialmente con el cáncer (De Felice et al., 2020; Wang X et al., 2021; Hasegawa et al., 2021). En este contexto, nosotros hemos querido contribuir a la filiación celular del carcinoma de células de Merkel estudiando la expresión en él de PIEZO2. Los resultados están contenidos en el trabajo titulado *Merkel cell carcinoma display PIEZO2 immunoreactivity* (J Persnalized Med. 2022; 12:894). Igualmente quisimos ver que sucede con la expresión de PIEZO2, y con la estructura y patrón proteico de los corpúsculos sensitivos y células de Merkel, en sujetos con diabetes mellitus tipo II que presentan neuropatía dolorosa y no dolorosa. Estudios previos han descrito anomalías en los corpúsculos sensitivos en condiciones de hiperglucemia (Paré M et al., 2007). Los resultados son la base del trabajo *Involvement of cutaneous sensory corpuscles in non-painful and painful diabetic neuropathy* (J Clin Med. 2021; 10:4609).

Por último, ante la aparición en los dos últimos años de evidencias sobre la expresión y funcionalidad de las proteínas PIEZO en tejidos no nerviosos, y ante la escasez o ausencia de datos en humanos, se ha realizado un mapeo de la expresión de PIEZO2, y ocasionalmente de PIEZO1, en diferentes tejidos humanos.

Los resultados obtenidos hasta la fecha han dado origen a un manuscrito que actualmente se encuentra en proceso de aceptación. Se titula PIEZO*1 AND PIEZO2 IN*

HUMAN MALE AND FEMALE GENITOURINARY SYSTEMS. An immunohistoclemical study (García-Mesa et al., *J Clin Med, enviado.*).

Los trabajos que se incluyen en el cuerpo de esta tesis, por orden cronológico de publicación son los siguientes:

1.- García-Mesa Y, García-Piqueras J, García B, Feito J, Cabo R, Cobo J, Vega JA, García-Suárez O. Merkel cells and Meissner's corpuscles in human digital skin display Piezo2 immunoreactivity. J Anat. 2017; 231: 978-989. doi: 10.1111/joa.12688.

2.- García-Mesa Y, García-Piqueras J, Cobo R, Martín-Cruces J, Suazo I, García-Suárez O, Feito J, Vega JA. Sensory innervation of the human male prepuce: Meissner's corpuscles predominate. J Anat. 2021; 239:892-902. doi: 10.1111/joa.13481.

3.- García-Mesa Y, Cárcaba L, Coronado C, Cobo R, Martín-Cruces J, García-Piqueras J, Feito J, García-Suárez O, Vega JA. Glans clitoris innervation: PIEZO2 and sexual mechanosensitivity. J Anat. 2021; 238:446-454. doi: 10.1111/joa.13317.

4.- García-Mesa Y, Feito J, González-Gay M, Martínez I, García-Piqueras J, Martín-Cruces J, Viña E, Cobo T, García-Suárez O. Involvement of Cutaneous Sensory Corpuscles in Non-Painful and Painful Diabetic Neuropathy. J Clin Med. 2021; 10:4609. doi: 10.3390/jcm10194609.

5.- García-Mesa Y, Feito J, Cuendias P, García-Piqueras J, Germanà A, García-Suárez O, Martín-Biedma B, Vega JA. The acquisition of mechanoreceptive competence by human digital Merkel cells and sensory corpuscles during development: An immunohistochemical study of PIEZO2. Ann Anat. 2022; 243:151953. doi: 10.1016/j.aanat.2022.151953.

6.- García-Mesa Y, García-Piqueras J, Cuendias P, Cobo R, Martín-Cruces J, Feito J, García-Suarez O, Biedma BM, Vega JA. Synaptophysin is a selective marker for axons in human cutaneous end organ complexes. Ann Anat. 2022; 243:151955. doi: 10.1016/j.aanat.2022.151955.

7.- García-Mesa Y, Martín-Sanz R, García-Piqueras J, Cobo R, Muñoz-Bravo S, García-Suárez O, Martín-Biedma B, Vega JA, Feito J. Merkel Cell Carcinoma Display PIEZO2 Immunoreactivity. J Pers Med. 2022; 12(6):894. doi: 10.3390/jpm12060894.

8.- García-Mesa Y, Cuendias P, M. Gago A, Feito J, Vega José A., García-Suárez O. PIEZO1 AND PIEZO2 IN HUMAN MALE AND FEMALE GENITOURINARY SYSTEMS. An immunohistochemical study. J Clin Med. Enviado.

El presente trabajo de tesis doctoral se ha realizado mediante la compilación de **ocho trabajos de investigación** que recogen todos los aspectos comentados en los párrafos precedentes. Además de la **Introducción**, en el volumen de la tesis doctoral se incluye una **Hipótesis de trabajo** y **Objetivos,** así como un apartado de **Discusión** general de los diferentes aspectos abordados en la tesis, y las **Conclusiones** del trabajo. Los **Resultados** son los propios trabajos que conforman la tesis. No se incluye un capítulo de **Material y Técnicas** porque son específicas para cada trabajo y en ellos se describen con el suficiente detalle como para ser replicados por cualquier otro investigador. Por otro lado, en el Anexo II de la tesis se adjuntan una serie de trabajos de investigación publicados en revistas internaciones en las que la autora de la tesis ha participado de forma activa durante el periodo de formación doctoral y de los que es coautora, y que no forman parte de la tesis.

El trabajo que se presenta como Tesis Doctoral cumple el Artículo 28 (*Presentación de la tesis como compendio de publicaciones*) del Reglamento de los Estudios de Doctorado aprobado el 20 de julio de 2018, del Consejo de Gobierno de la Universidad de Oviedo y publicados en Boletín Oficial del Principado de Asturias, núm. 185 de 9-viii-2018.

2. Hipótesis y Objetivos

Hipótesis

En base al planteamiento general del estudio se han establecido tres hipótesis de trabajo:

Hipótesis 1

PIEZO2 es un canal activado mecánicamente requerido para la discriminación táctil y la propiocepción, así como otras variedades de mecanosensibilidad. Teniendo en cuenta que la mecanotransducción para ese tipo de sensibilidades se lleva a cabo en órganos sensitivos cutáneos complejos (OSCC), cabe esperar que alguno de los componentes celulares de los mismos exprese PIEZO2.

Hipótesis 2

Sobre la base de que PIEZO2 es el responsable de la mecanotransducción en todos los sistemas biológicos deben existir células PIEZO2 positivas en todos los tejidos mecanosensibles.

Hipótesis 3

La expresión de tisular de PIEZO2 está alterada en patologías que cursan con alteraciones de la sensibilidad, mecánica o no, y es positivo en los tumores derivados de células que expresan PIEZO2.

Objetivos

El **objetivo general** del estudio es contribuir al conocimiento de la mecanobiología mediada por el canal PIEZO2 en órganos sensitivos cutáneos complejos y en tejido no nerviosos, tanto en condiciones de normalidad como patológicas.

Los **objetivos específicos** son:

1.- Estudiar la presencia y localización del canal PIEZO2 en la piel glabra humana (piel digital, del prepucio y del clítoris), con especial atención a los órganos sensitivos cutáneos complejos (corpúsculos de Meissner y Pacini) y complejos célula de Merkel-neurita.

2.- Establecer el momento del desarrollo embrionario en el que los órganos sensitivos cutáneos complejos y los complejos células de Merkel-neurita de la piel glabra de los

dedos comienzan a expresar PIEZO2 como indicador de la adquisición de competencia para transducir estímulos mecánicos.

3.- Identificar las células mecanosensibles en el sistema genito-urinario humano en base a la expresión de PIEZO2.

4.- Analizar los cambios en la expresión de PIEZO2 por los órganos sensitivos cutáneos complejos y los complejos células de Merkel-neurita de la piel glabra en el curso de la neuropatía diabética.

5.- Estudiar si los tumores derivados de células PIEZO2 positivas mantienen o alteran los patrones de expresión del mismo durante la transformación tumoral; se utiliza como paradigma el Merkeloma cutáneo de cabeza y cuello.

3. Resultados

3.1. Resultados – Publicación 1

Annals of Anatomy. 243 (2022): 15/955.

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Research article

Synaptophysin is a selective marker for axons in human cutaneous end organ complexes

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ABSTRACT

Background: Small clear synaptic-like vesicles fill axon terminals of mechanoreceptors. Their functional significance is controversial and probably includes release of neurotransmitters from afferent axon terminals. Synaptophysin, a major protein of the synaptic vesicle membrane, is present in presynaptic endings of the central and peripheral nervous systems. It is also expressed in mechanosensory neurons which extend into skin forming sensory corpuscles. Nevertheless, synaptophysin occurrence in these structures has never been investigated.

Methods: Here we used immunohistochemistry to detect synaptophysin in adult human dorsal root ganglia, cutaneous Meissner and Pacinian corpuscles and Merkel cell-neurite complexes from foetal to elderly period. Moreover, we analyzed whether synaptophysin co-localizes with the mechano-gated protein PIEZO2.

Results: Synaptophysin immunoreactivity was observed in primary sensory neurons (36 ± 6%) covering the entire soma size ranges. Axons of Meissner's and Pacinian corpuscles were positive for synaptophysin from 36 and 12 weeks of estimated gestational age respectively, to 72 years old. Synaptophysin was also detected in Merkel cells (from 14 weeks of estimated gestational age to old age). Additionally in adult skin, synaptophysin and PIEZO2 co-localized in the axon of Meissner and Pacinian corpuscles, Merkel cells as well as ~~in~~ some axons of Merkel cell-neurite complexes.

Conclusion: Present results demonstrate that a subpopulation of primary sensory neurons and their axon terminals forming cutaneous sensory corpuscles contain synaptophysin, a typical presynaptic vesicle protein. Although the functional relevance of these findings is unknown it might be related to neurotransmission mechanisms linked to mechanotransduction.

1. Introduction

Synaptophysin (SYN), also known as protein p38, is a major integral glycoprotein of the synaptic vesicle membrane encompassing the 8% of total protein (Valtorta et al., 2004; Evans and Cousin, 2005). It is involved in synaptic vesicle formation and recycling (Alder et al., 1992, 1995; Shibaguchi et al., 2000; Daly and Ziff, 2002; Horikawa et al., 2002), synapse formation (Tarsa and Goda, 2002) and synaptic transmission (Valtorta et al., 2004; Kovacs, 2017). Consistently with these functions, SYN is supposedly present at all presynaptic endings and widely distributed in all brain regions (De Camilli et al., 1988). In the peripheral nervous system, SYN has been detected in synaptic profiles of sympathetic and parasympathetic ganglia (Diaz and Diana, 1992; Luckensmeyer and Keast, 1995; Roudenok and Kühnel, 2002), as well as in the enteric nervous system (see Parathan et al., 2020). SYN in rat is also expressed in about ~~the~~ 80% of total DRG neurons ~~in rat~~ (including all large neurons and Aβ-fibers) and afferent fibers to III–IV laminae of spinal

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0940-9602/

dorsal horn (Sun et al., 2006). Most large primary sensory neurons/Aβ-fibers work as low-threshold mechanoreceptors (LTMRs) (Abraira and Ginty, 2013), which extends into skin to form sensory corpuscles or cutaneous end organ complexes (CEOC; Handler and Ginty, 2021). In fact, axons of LTMRs in dermis are associated with either specialized terminal glial cells forming sensory corpuscles (Meissner, Ruffini, and Pacinian corpuscles), either specialized epithelial cells (Merkel cells) forming Merkel cell-axon complexes, or hair follicles cells forming piloneural complexes (Rice and Albercht, 2008; Zimmerman et al., 2014; Cobo et al., 2021; Suazo et al., 2022).

Since LTMRs expressing SYN project into skin, it might be hypothesized that axon terminals of CEOC contain SYN. According to this supposition, Bewick (2015) conducted a superb review highlighting the occurrence of synaptic-like vesicles, synaptic vesicle-associated proteins (including synaptophysin), Ca^{2+}-binding proteins, and a synaptic glutamatergic system in axon terminals of mechanosensory afferents of muscle spindle and lanceolate endings from hairy skin. Furthermore, clear synaptic-like vesicles have been found in axon terminals supplying Meissner and Pacinian corpuscles by using transmission electron microscopy (see Munger and Idé, 1988; Zelená, 1994), as well as reported indirect evidence for glutamatergic neurotransmission in Pacinian corpuscles (Pawson et al., 2007, 2009). Varga et al. (2020) have also recently observed SYN-immunoreactivity in the axon of Pacinian corpuscles.

Interestingly, synaptic-like vesicles in mechanosensory terminals are functionally linked to ion channels involved in mechanotransduction, like degenerin-epithelial Na^+ channel (DEG/ENa$^+$C) subunits or acid-sensing ion channels (ASIC; see for a review Cobo et al., 2020). However, as far as we know the only ion channel that fulfills conditions to be considered as a mechanotransducer is PIEZO2 (Coste et al., 2010), which is present in CEOC (Cobo et al., 2020) including human Merkel cell-neurite complexes, and Meissner and Pacinian corpuscles (García-Mesa et al., 2017; García-Piqueras et al., 2019).

Therefore, the present study was designed to investigate SYN occurrence in the human cutaneous sensory corpuscles and Merkel cell-axon complexes and analyse age-related changes from prenatal to elderly life periods. Moreover, co-localization between SYN and the mechanotransducer ion channel PIEZO2 was examined.

2. Material and Methods

2.1. Materials and tissue treatment

Experiments were conducted on skin samples from the palmar side of the distal phalanx, belonging to the histological collection of SINPOs Research Group at the University of Oviedo (Registro Nacional de Biobancos, Sección colecciones, Ref. C-0001627). Skin pieces were obtained from amputated fingers (n = 12) or necropsies of free neurological-disease subjects (n = 8) within 6–12 h after accident or death at the Department of Pathology of HUCA (Oviedo, Spain). The age range of those subjects was 8–72 years old. Furthermore, same-location skin samples were extracted from either amputated supernumerary fingers (n = 3) or fingers obtained during autopsy of fetuses and perinatally deceased children (n = 34). Fetuses and children age was ranged from 11 WEGA (weeks of estimated gestational age) to 3 years old. These materials proceeded from the Departments of Pathology of Hospital Universitario Central de Asturias (HUCA, Oviedo, Spain), Hospital Universitario Donostia (San Sebastián, Spain), Hospital José Molina Orosa (Lanzarote, Spain) and Complejo Hospitalario Universitario de Salamanca (Salamanca, Spain). On the other hand, human lumbar dorsal root ganglia (DRG; n = 8) were obtained from 4 healthy adult males who died in traffic accidents (age-ranged from 35 to 56 years old), during organ removal for transplantation (HUCA, Oviedo, Spain).

All used tissues were obtained in compliance with the Spanish Law (RD 1301/2006; Ley 14/2007; DR 1716/2011; Orden ECC 1414/2013) and the study was approved by the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. CElm, PAst: Proyecto 266/18).

Specimens were fixed in 4% formaldehyde in 0.1 M phosphate buffer saline (pH 7.4) for 24 h, dehydrated and embedded in paraffin. Paraffin-embedded tissues were cut into 10-μm-thick sections perpendicularly to skin surface and mounted on gelatine-coated microscope slides. Presence of sensory corpuscles in skin samples was ensured using haematoxylin-eosin staining.

2.2. Immunohistochemistry

Deparaffinized and rehydrated sections were processed for SYN immunodetection using EnVision antibody complex detection kit (DakoCytomation, Copenhagen, Denmark) and following supplier's instructions. Briefly, endogenous peroxidase activity and non-specific binding were blocked, and sections were then incubated with a mouse monoclonal antibody against SYN (1:200 dilution; clone DAK-SYNAP; DAKO, Glostrup, Denmark; reference M7315) overnight at 4 °C. Subsequently, sections were rinsed, and incubated with Dako EnVision System labeled polymer-HR anti-mouse IgG (DakoCytomation) for 30 min at room temperature. Finally, sections were washed, and immunoreaction visualized using 3–3′-diaminobenzidine as a chromogen. To ascertain structural details, some sections were counterstained with Mayer's haematoxylin, dehydrated, and mounted with Entellan® (Merk, Dramstadt, Germany).

2.3. Double immunofluorescence

Sections were processed for simultaneous detection of SYN and neurofilament proteins (NFP; polyclonal, raised in rabbit; Invitrogen, Massachusetts, USA; reference ab204893, EPR20020), S100 protein (S100P; polyclonal, raised in rabbit; DAKO, Glostrup, Denmark; reference IS504), or PIEZO2 (polyclonal, raised in rabbit; Sigma Aldrich, Saint Louis, MO, USA; reference PA5–72976, amino acid sequence FEDEN-KAAVRIMAGDNVEICMNLDAASFSQHNP). NFP and S100P label axons and lamellar cells respectively, within sensory corpuscles (Vega et al., 2009; Cobo et al., 2021).

Double immunostaining was performed on 10-μm-thick deparaffinized and rehydrated sections. Non-specific binding was reduced by incubation with a solution of 1% bovine serum albumin in tris buffer solution (TBS) for 30 min. Sections were later incubated with a 1:1 mixture of mouse anti-SYN and rabbit anti-NFP antibodies (both 1:100 diluted in the blocking solution), or mouse anti-SYN and rabbit anti-S100 protein antibodies (1:100 and 1:1000 diluted respectively), or mouse anti-SYN and rabbit anti-PIEZO2 antibodies (1:100 and 1:200 diluted respectively) overnight at 4ºC in a humid chamber. After rinsing with TBS, slides were incubated with Alexa fluor 488-conjugated goat anti-rabbit IgG (1:100 diluted in TBS containing 5% mouse serum; Serotec, Oxford, UK), then rinsed again and incubated with Cy™3-conjugated donkey anti-mouse antibody (1:100 diluted in TBS; Jackson-ImmunoResearch, Baltimore, MD, USA) for 1 h. Both steps were performed at room temperature in a dark humid chamber. Finally, sections were counterstained with DAPI (10 ng/ml) to label the nuclei, washed, dehydrated and mounted with Fluoromount Gold (ThermoFisher, Runcoen, UK).

Double immunofluorescence was detected using a Leica DMR-XA automatic fluorescence microscope coupled with a Leica Confocal Software (version 2.5; Leica Microsystems, Heidelberg GmbH, Germany), and captured images were processed using Image J software (version 1.43 g; Master Biophotonics Facility, Mac Master University Ontario; www.macbiophotonics.ca).

For control purposes, representative sections were processed in the same way as described above but either using non-immune

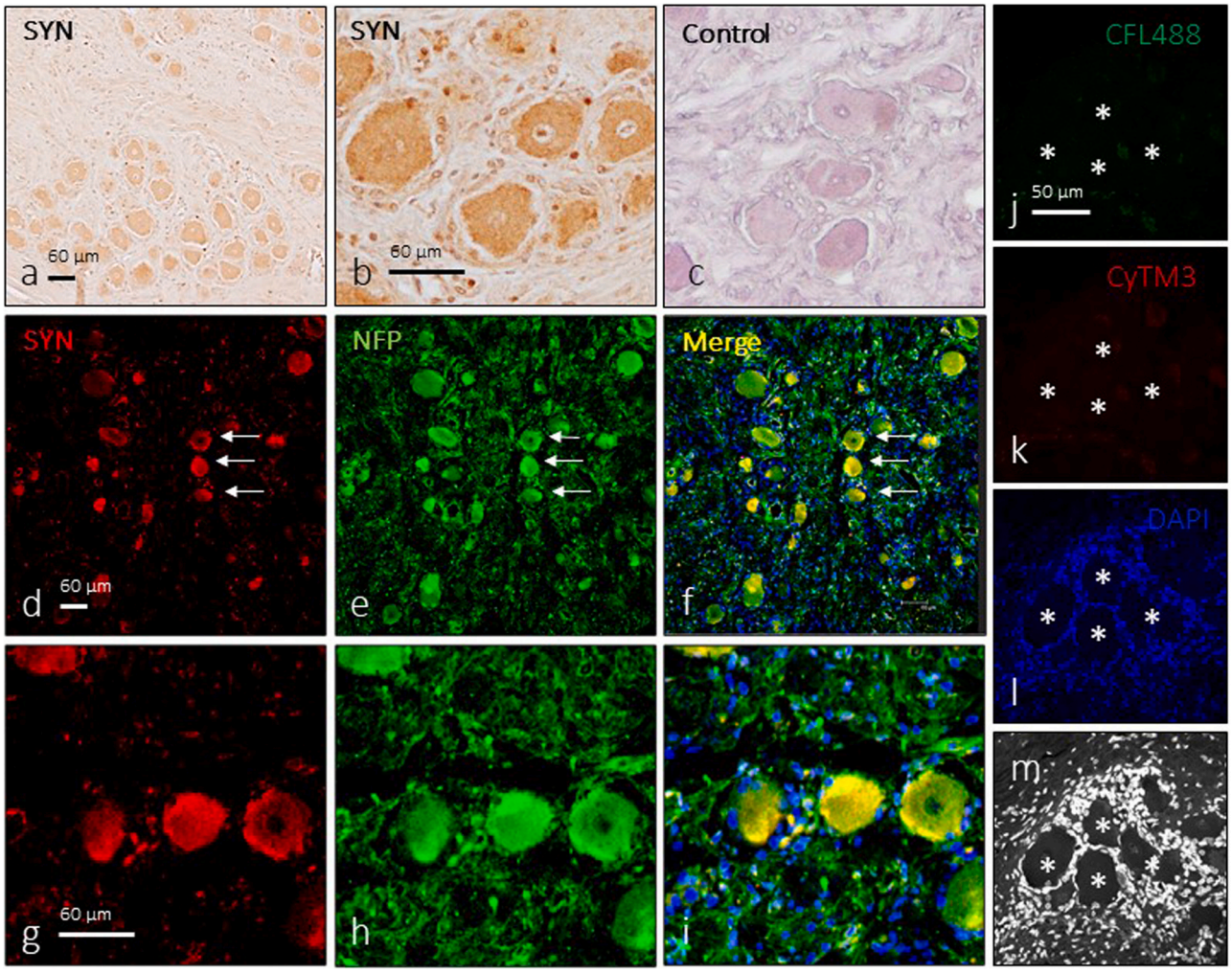

Fig. 1. Immunohistochemical detection of synaptophysin in human lumbar dorsal root ganglia (**a**,**b**) Double immunofluorescence for synaptophysin (SYN, red; **d** and **g**) and neurofilament proteins (NFP, green; **e** and **h**) in human lumbar dorsal root ganglia. SYN co-localized with NFP (yellow; **f** and **i**) in the soma of DRG primary sensory neurons of different sizes. In sections in which the primary antibody was omitted neither immunoreactivity nor immunofluorescence (**j-m**) was observed. **d-f** objective 40X/1.25 oil, pinhole 1.00, XY resolution 156 nm and Z resolution 334 nm; **g-i** objective 63X/1.40 oil, pinhole 1.37, XY resolution 139.4 nm and Z resolution 235.8 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rabbit or mouse sera instead of primary antibodies or omitting primary antibodies when incubation. No positive immunostaining was observed under these conditions (data not shown).

2.4. Quantitative study

A quantitative image analysis was carried out in adult SYN-positive DRG using an automatic image analysis system (Quantimet 550, Leika, QWIN Program). Percentage and size (mean diameter in µm) of immunoreactive neurons were evaluated. Measurements were made on three sections per specimen, 200 µm apart between them to avoid measure the same neuron twice, evaluating five randomly selected fields per section (2.5 mm^2). For evaluation of cell body size just neuronal profiles with apparent nuclei were considered, and neurons were divided into 3 size classes according to their diameter: ≤ 20 µm (small neurons), 21–50 µm (intermediate neurons), and > 50 µm (large neurons).

Moreover, percentage of Meissner and Pacinian corpuscles displaying SYN immunoreactivity was calculated in 10 sections per specimen, 100 µm apart. Total number of sensory corpuscles in an entire section was determined by counting the number of sensory corpuscles displaying S100P-immunoreactivity (for details see García-Piqueras et al., 2019).

3. Results

3.1. Dorsal root ganglia

The first step of this research was to investigate SYN occurrence in DRG. SYN was detected in neurons of all analyzed samples, covering the entire soma area (Fig. 1a and d), as well as in intraganglionic axon profiles (Fig. 1a-b; 1d and 1 g). These SYN-positive neurons were too immunoreactive for NFP (Fig. 1e and h), and both proteins co-localized (Fig. 1f and i). Within the neuronal body, the immunostaining pattern was cytoplasmic, and sometimes observed more remarkably on the periphery, close to the plasma membrane. Contrary, satellite glial cells lacked SYN-immunoreactivity. SYN-positive neuronal bodies were about 36 ± 6% (242/672 evaluations) of total counted neurons and distributed ~~in~~ across the above preestablished size-categories as follows: 11% small neurons (74/672), 23% intermediate neurons (155/672), and 2% (13/672) large neurons. No immunoreactivity nor immunofluorescence for SYN was observed in control sections (Fig. 1c, j-m).

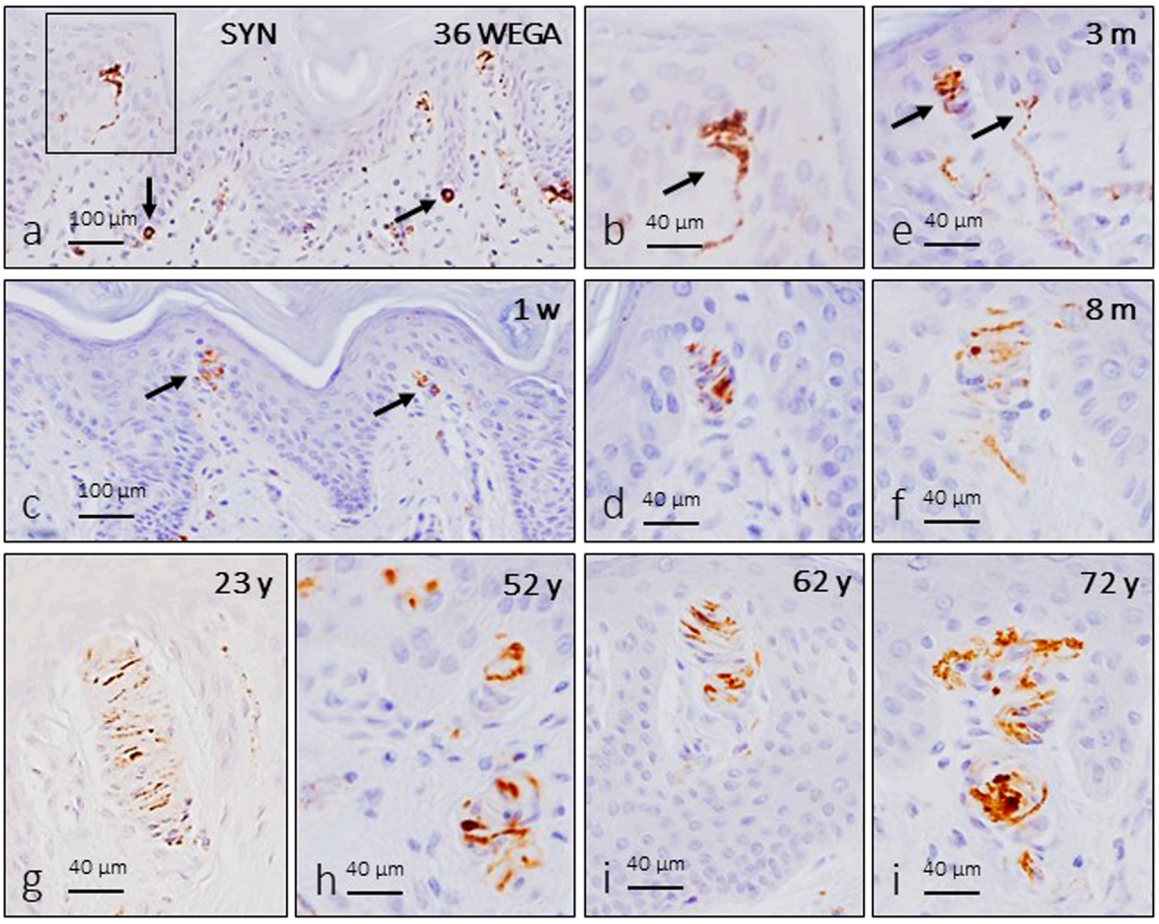

Fig. 2. Immunohistochemical detection of synaptophysin in axonal profiles of Meissner corpuscles in the digital skin at different ages: 36 WEGA (**a**, **b**), 1 week (**c**, d), 3 months (**e**), 8 months (**f**), and 23, 52, 62 and 72 years (**g** to **i**, respectively). Synaptophysin-positive Merkel cells were detected (arrows in **a**).

3.2. Meissner's corpuscles

Axonal profiles of Meissner's corpuscles were positive for SYN independently of age, from very early (36 weeks WEGA) to 72 years old (Fig. 2). Immunohistochemistry for NFP and SYN in consecutive sections suggested axonal localization of SYN in Meissner's corpuscles (Fig. 3). To confirm that, double immunofluorescence was performed (Fig. 4): SYN was shown to co-localize with NFP (Fig. 4a-d) but no with S100P (Figs. e-g). However, a few Meissner's corpuscles seemed to have different distribution of SYN-NFP along their axonal terminal, showing a preference of SYN for the corpuscular apical pole, opposite to the basal NFP immunostaining (Fig. 4d).

Regarding sampled subject ages, the number of Meissner corpuscles displaying SYN immunoreactivity was as follows: 81 ± 11% at 36 WEGA, 88 ± 9% at 1 week, 79 88 ± 6% from 8 to 10 months, 100% from 8 to 65 years old, 76 ± 14% over 65 years old. Thus, no significant age-dependent differences were noted.

3.3. Pacinian corpuscles

As well as in Meissner's corpuscles, SYN immunoreactivity was visualized in the axon of all identified Pacini's corpuscles. Despite Pacinian corpuscles starting development at 12 WEGA, ~~and~~ the first SYN occurrence in their axon was detected at 18 WEGA, and then retained over all studied time periods (Fig. 5) in the 100% of ~~the~~ Pacinian corpuscles. Although SYN distribution pattern within the corpuscle was clearly axonal, double immunofluorescence for NFP and SYN verified this, since both co-localized (Fig. 5i).

3.4. Merkel cell-neurite complexes

SYN was also detected in Merkel cells and apparently, in some axonal profiles contacting them (Fig. 6). SYN-positive structures were observed unequally distributed between the most external cells of immature epidermis at 14 WEGA (Fig. 6a-b). Once the epidermis acquires its multilayered structure (between 22 and 36 WEGA), clusters of SYN-positive cells appeared in the basal layer (Fig. 6c-f). In adult tissues, SYN was detected in isolated cells within the basal layer of the epidermis (Fig. 6g-i) forming immunoreactive rings, which were identified as Merkel cells; this typical immunohistochemical pattern seemed to become disorganised in the elderly (Fig. 6i). Definitive identification of those epidermal cells as Merkel cells was based on co-localization between SYN and CK-20 (data not shown).

In regard to sample ages, percentage of Merkel cells displaying SYN immunoreactivity (considering CK20-positive cells as a reference) was as follows: 93 ± 11% at 22 WEGA, 85 ± 9% at 36 WEGA, 72 ± 12% at 1 week, 74 ± 8% from 8 to 10 months, 72 ± 4% at 3 years old, 49 ± 6% at 23 years old, 51 ± 7 at 45 years old, and 41 ± 11% over 65 years old. Thus, density of SYN-positive Merkel cells was higher during fetal life first postnatal years, followed by a progressive decrease with age.

3.5. SYN and PIEZO2 co-localize in Meisner corpuscles, Pacinian corpuscles, and Merkel cells

Since synaptic-like vesicles and mechanosensory axon terminals are functionally related to ion channel participating in

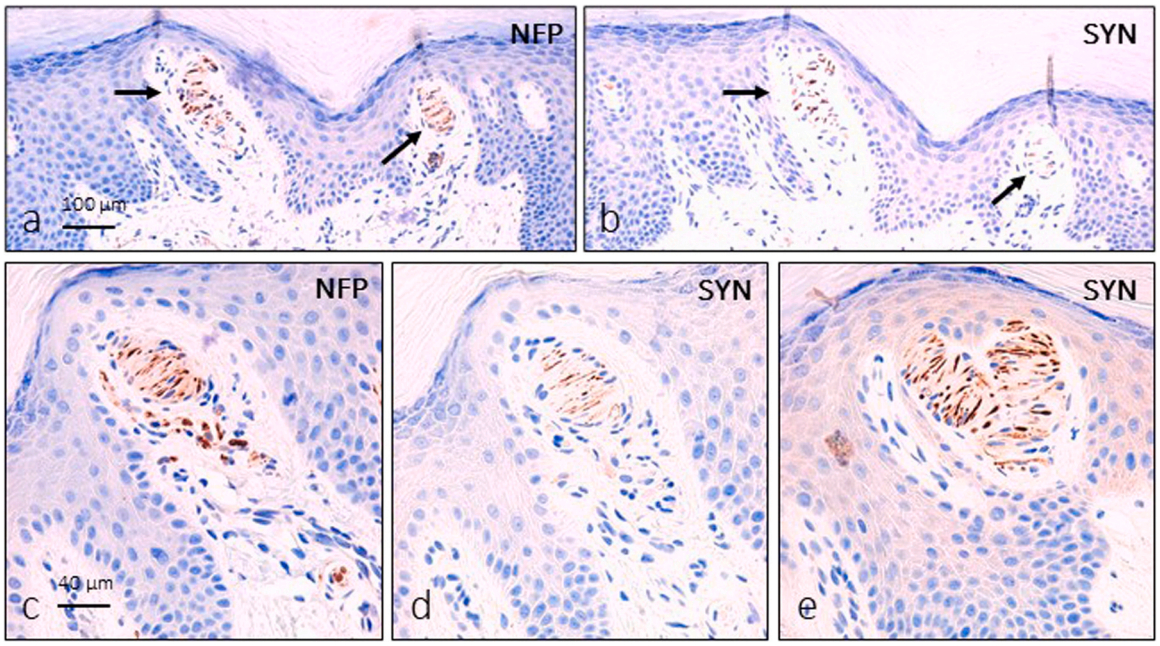

Fig. 3. Immunohistochemical detection of synaptophysin (SYN) and neurofilament proteins (NFP) in serial sections (**a** and **b**; **c** and **d**) of Meissner corpuscles of digital skin, corresponding to one subject 23 years old (**e**), 35 years old (a and b) and one subject 48 years old. Both synaptophysin and NFP show similar patterns of localization.

mechanotransduction, we analyzed whether SYN co-localizes with the mechanogated ion channel PIEZO2 in axon terminals of sensory corpuscles. Using double immunofluorescence for SYN and PIEZO2 we observed partial co-localization in axons of Meissner (Fig. 7a-c) and Pacinian corpuscles (Fig. 7f). Despite full co-localization between SYN and PIEZO2 in some Merkel cells (Fig. 7d), some others lacked PIEZO2 immunoreactivity, in which case it was observed in axons supplying them (Fig. 7e).

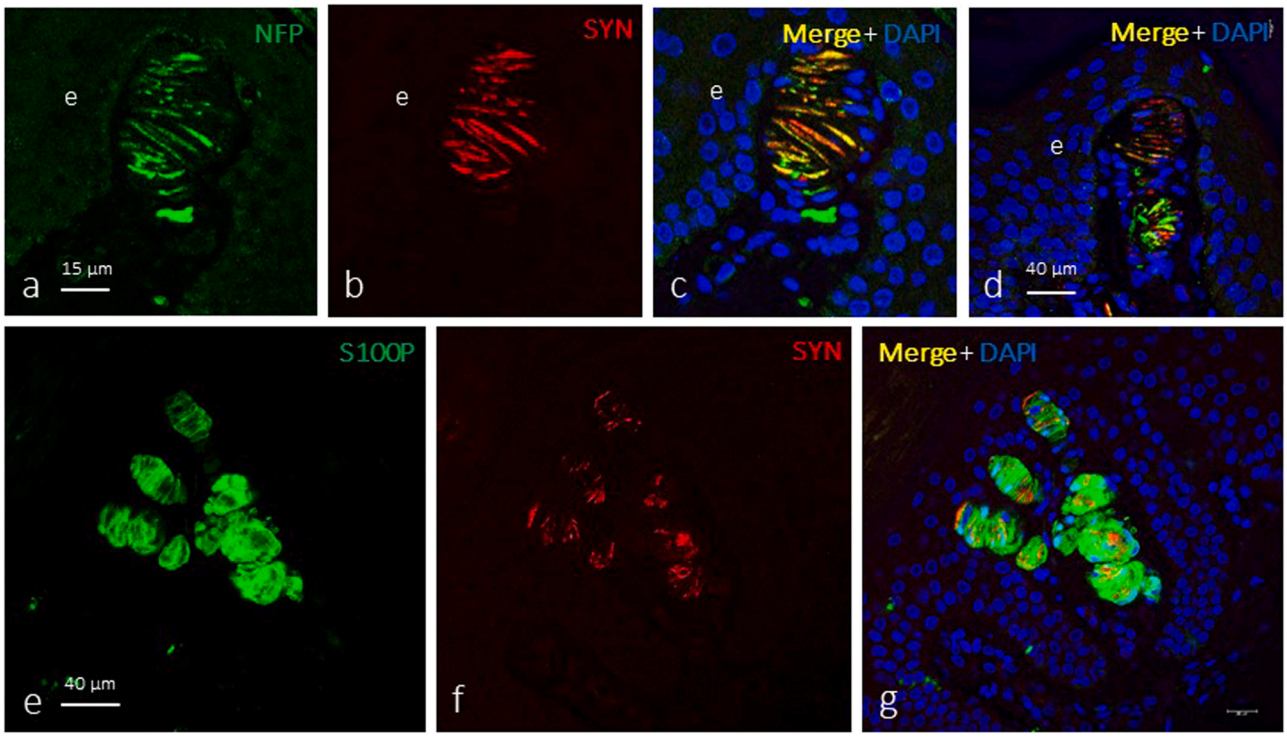

Fig. 4. Confocal dual immunofluorescence for synaptophysin (SYN, red) and neurofilament proteins (NFP) or S100 protein (S100P, green) in human digital Meissner's corpuscles. SYN totally or in part co-localize with NFP (**a-c** and **d**) but did not co-localize with S100 protein (**e** to **g**) when merged (**c**, **d** and **9**) but. **a-c** objective 63X/1.40 oil, pinhole 1.37, XY resolution 139.4 nm and Z resolution 235.8 nm; **d-e** and **g** objective 40X/1.25 oil, pinhole 1.00, XY resolution 156 nm and Z resolution 334 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

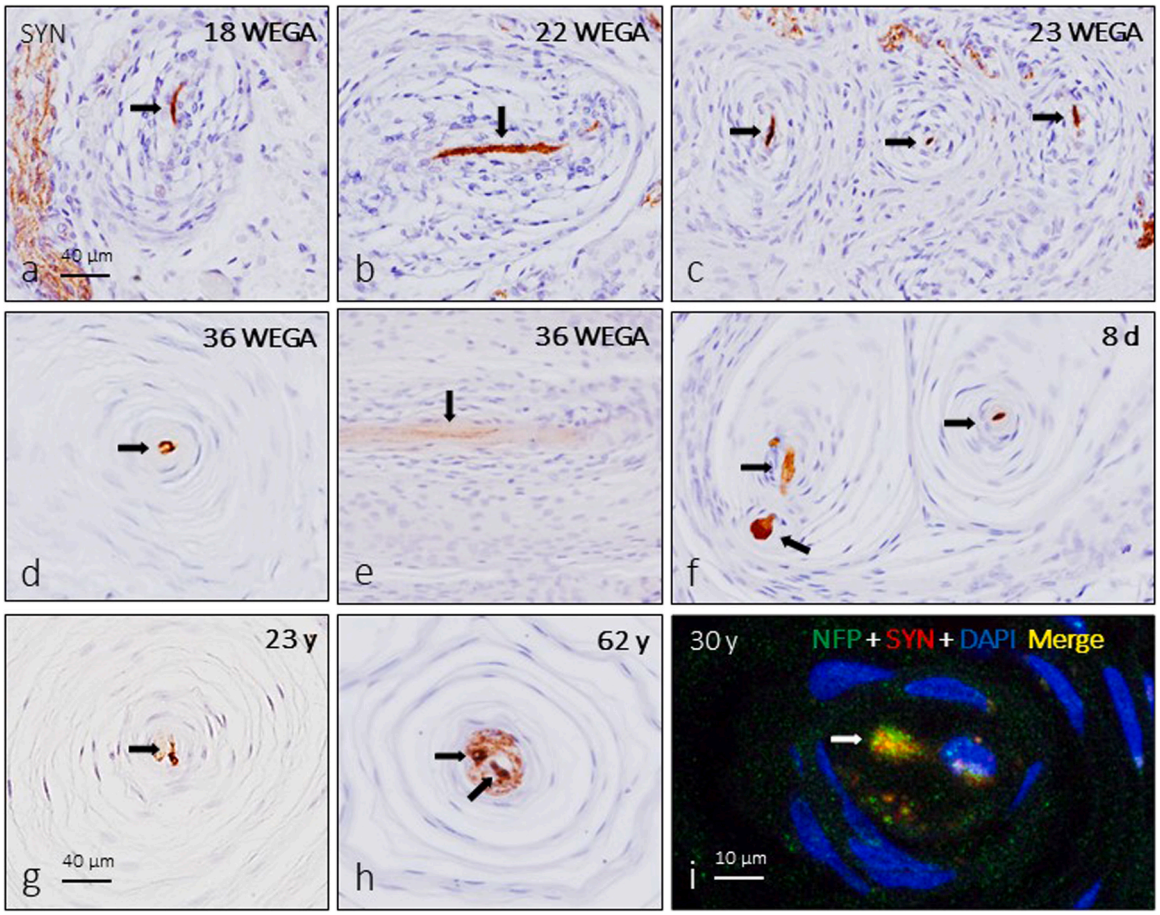

Fig. 5. Immunohistochemical detection of synaptophysin (SYN) in human cutaneous corpuscles of Pacini at different ages: 18, 22, 23 and 36 WEGA (**a** to **e**), 8 days (**f**) 23 years (**g**) and 62 years (**h**). SYN was found in the axon of Pacinian corpuscles (arrows in a to h) at all studied ages. Picture in **i** shows confocal dual immunofluorescence for SYN (red) and NFP (green) showing the co-localization of both proteins in the axon. Objective 63X/1.40 oil, pinhole 1.37, XY resolution 139.4 nm and Z resolution 235.8 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Other cutaneous structures

SYN immunoreactivity was also detected in nerve plexuses surrounding dermal blood vessels (Fig. 8a) and sweat glands (Fig. 8b-e). In nerve trunks (Fig. 7d and e), SYN immunofluorescence was detected in a subpopulation of axons, preferentially those large and surrounded by Schwann cells, which demonstrated the same organized structure than in sensory corpuscles (Fig. 7f).

4. Discussion

The present research was aimed to investigate the occurrence of SYN, a synaptic vesicle membrane protein (Valtorta et al., 2004) virtually present in all presynaptic terminals (De Camilli et al., 1988), in both human DRG and ~~the~~ axon terminals of CEOC. Secondly, co-localization of SYN with the mechanotransducer ion channel PIEZO2 was studied. The research covers a wide age-range (from 11 WEGA to 72 years old) and was performed using immunohistochemistry and double immunofluorescence.

The motivation to investigate this topic was based on the following facts: a) SYN expression in DRG, including LTMRs (Sun et al., 2006); b) sensory corpuscle axon terminals are typically filled with small clear synaptic-like vesicles (see for review Malinovský and Pack, 1982; Munger and Idé, 1988; Zelená, 1994); c) SYN occurrence in afferent nerve endings of muscle spindles and tendon sensory organs (De Camili et al., 1988), as well as in Pacinian corpuscles (Varga et al., 2020); and, d) PIEZO2 expression in axons of human CEOC (García-Mesa et al., 2017).

No information exists about the presence of SYN in human DRG. Our results on primary sensory neurons basically agree with previous studies conducted in rat DRGs by Sun et al. (2006). Here we report that approximately the 25% of neurons, including intermediate and large size ranges, were SYN-positive. Considering that axon terminals of these neurons form CEOCs on the periphery (Li et al., 2011; Abraira and Ginty, 2013; Handler and Ginty, 2021), some of them (if not all) are expected to display axonal SYN immunoreactivity. According to this supposition, our findings on human digital skin demonstrate that axon terminals of Pacini's and Meissner corpuscles show SYN immunoreactivity at all examined ages, from fetal period to old age. Therefore, we conclude that SYN presence in axons of these two types of CEOC is a common and permanent characteristic. On the other hand, although SYN-positive spinal terminals from rat DRG display morphological characteristics of endings of myelinated primary mechanosensitive afferents rather than nociceptive unmyelinated afferents (Lu et al., 2003), these findings were not confirmed and SYN was detected in both Aβ and Aδ/C spinal terminals (Chung et al., 2019).

A particularity of LTMR terminal axons is their capability to form Merkel-neurite cell complexes, since they contact with differentiated specialized epithelial cells (i.e., Merkel cells; Zimmerman et al., 2014; Cobo et al., 2021), but do not with specialized peripheral glial cells (the so-called terminal glia; Reed et al., 2021). Merkel cells contain synapse-like vesicles at their cytoplasmic axonal pole (Moll et al. 2005; Boulais and Missery, 2007), which expresses SYN immunoreactivity (Ortonne et al., 1988; see Maksimovic et al., 2013). Our results show that SYN is present in Merkel cell cytoplasm from

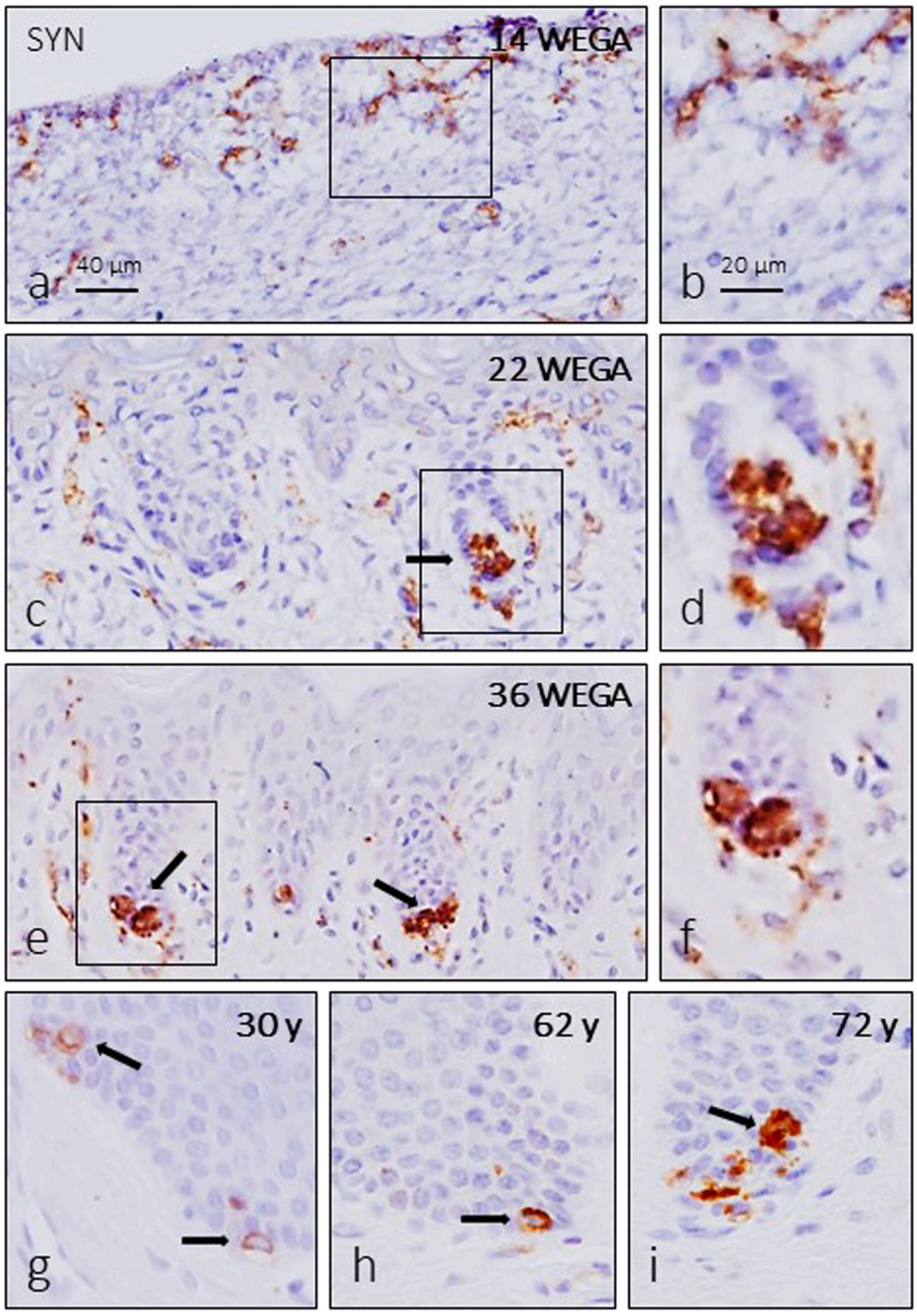

Fig. 6. Immunohistochemical detection of synaptophysin in human digital skin focusing on Merkel cells-neurite complexes at different ages: 14 WEGA (**a**, **b**), 22 WEGA (**c**, **d**; arrows), 36 WEGA (**e**, **f**; arrows). Individual Merkel cells in the basal layer of the epidermis were selectively SYN-positive in adults: 30 years (**g**), 62 years (**h**) and 72 years (**i**).

the beginning of their development ~~and~~ to throughout adult life, although not all Merkel cells express it in adult digital skin. Additionally, we have observed SYN immunoreactivity in some axon terminals contacting Merkel cells, thus confirming which confirms the occurrence of SYN in LTMR afferents.

The prompt observations of synaptic-like vesicles in CEOC axon terminals using electron microscopy had controversial interpretation about their role, including likely release of neurotransmitters (Malinovský and Pac, 1982; Munger and Idé, 1988; Zelená, 1994). De Camilli et al. (1988) suggested possible functional similarities between small synaptic vesicles at presynaptic nerve endings and small clear vesicles of CEOC, and Bewick et al. (2005) posteriorly affirmed that synaptic-like vesicles in sensory terminals of primary mechanosensory neurons resemble the synaptic vesicles of chemical synapses. Our findings on human CEOC and Merkel cell-neurite complexes lend support to a possible neurotransmission within these structures. A possible GABA-ergic/glutamatergic neurotransmission in Pacinian corpuscles (Pawson et al., 2007, 2009), palisade ending and muscle spindles (Bewick et al., 2005; Bewick, 2005) has been suggested, but this remains to be proven as a common characteristic of mechanoreceptors. In that case, the presence of synaptic-like vesicles in CEOC axon terminals might have an explanation.

It is now well known that Final del formulario mechanotransduction depends on the mechanical sensitivity of certain ion channels, which may be mechanically gated, or may show

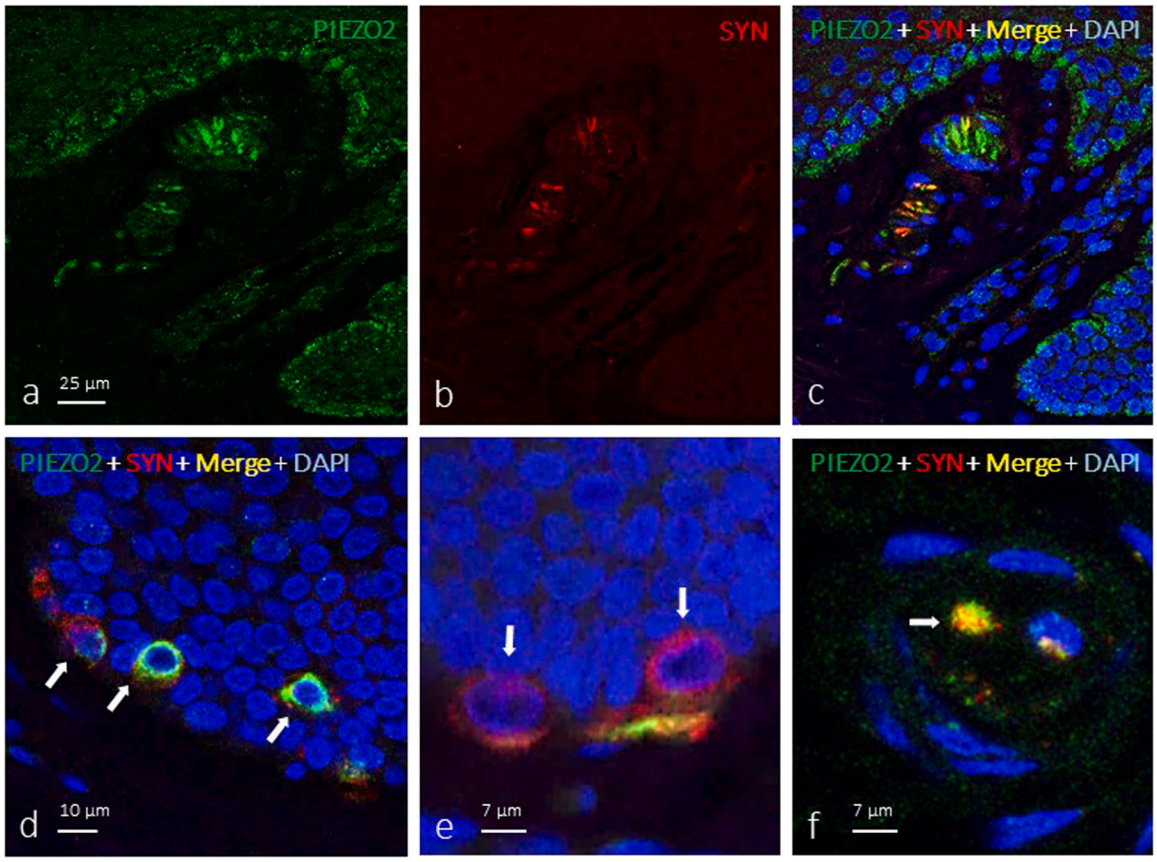

Fig. 7. Double immunofluorescence for synaptophysin (SYN, red) and PIEZO2 (green) in human cutaneous Meissner corpuscles (**a-c**), Merkel cell-neurite complexes (**d** and **e**), and Pacinian corpuscles (**f**). SYN and PIEZO2 co-localized partially and irregularly with PIEZO2 the axon of Meissner and Pacinian corpuscles. In Merkel cells PIEZO2 co-localized with SYN in most cases (arrows in d), but Merkel cells SYN positive and PIEZO2 negative were observed. In some cases, only the nerve afferent of the Merkel cell-neurite complexes showed co-localization (**e**). **a-c** objective 40X/1.25 oil, pinhole 1.00, XY resolution 156 nm and Z resolution 334 nm; **d-f** objective 63X/1.40 oil, pinhole 1.37, XY resolution 139.4 nm and Z resolution 235.8 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

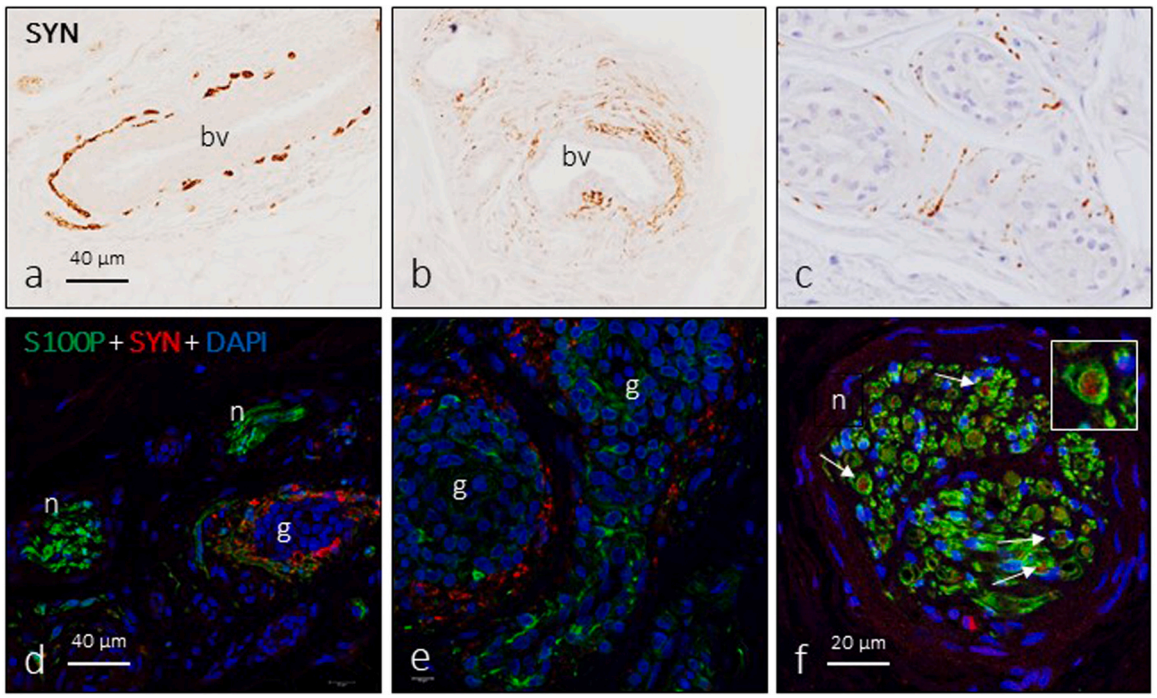

Fig. 8. Single immunohistochemistry (**a-c**) and double immunofluorescence (synaptophysin and S100 protein) for synaptophysin (SYN) in distinct anatomical structures of skin dermis. SYN was found in nerve profiles around blood vessels (bv; **a**, **b**) and sweat glands (g; **c**, **d**, **f**). In dermal nerve trunks large axons displayed SYN immunofluorescence (arrows in **f**). **d** and **e** objective 40X/1.25 oil, pinhole 1.00, XY resolution 156 nm and Z resolution 334 nm; **f** objective 63X/1.40 oil, pinhole 1.37, XY resolution 139.4 nm and Z resolution 235.8 nm.

mechanical sensitivity (see Cobo et al., 2020). To date, the only channel that fulfills the properties of a mechanotransducer is PIEZO2. In the present study, we have confirmed previous results from our lab reporting PIEZO2 immunodetection in different morphotypes of human CEOC and Merkel cell-neurite complexes (García-Mesa et al., 2017, 2021a,b; García-Piqueras et al., 2019). Furthermore, we observed that PIEZO2 and SYN fully co-localize in CEOC axon terminals. To the best of our knowledge, PIEZO2 is present in the axonal membrane but not in vesicles. Therefore, the functional significance of these findings, if any, remains to be demonstrated. SYN deficient mice are viable and do not show defects in neurotransmitter release (Eshkind and Leube, 1995; McMahon et al., 1996). In any case, our data suggest that SYN is a reliable marker for peripheral axon terminals of primary mechanosensory neurons forming CEOC.

Ethical statement

This study was approved by the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. CElm, PAst: Proyecto 266/18).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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3.2. Resultados – Publicación 2

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Yolanda García-Mesa, Jorge Feito, Patricia Cuendias, Jorge García-Piqueras, Antonino Germanà, Olivia García-Suárez, Benjamín Martín-Biedma, José A. Vega.

The acquisition of mechanoreceptive competence by human digital Merkel cells and sensory corpuscles during development: An immunohistochemical study of PIEZO2.

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The acquisition of mechanoreceptive competence by human digital Merkel cells and sensory corpuscles during development: An immunohistochemical study of PIEZO2

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ABSTRACT

Background: PIEZO2 is a transmembrane protein forming part of an ion channel required for mechanotransduction. In humans, PIEZO2 is present in axon terminals of adult Meissner and Pacinian corpuscles, as well as Merkel cells in Merkel cell-neurite complexes.
Methods: To study the acquisition of functional capability for mechanotransduction of developing type I slowly adapting low-threshold mechanoreceptors, i.e., Merkel cell-neurite complexes, a battery of immunohistochemical and immunofluorescence techniques was performed on human skin specimens covering the whole development and growth, from 11 weeks of estimated gestational age to 20 years of life. In addition, developmental expression of PIEZO2 type I (Meissner's corpuscles) and type II (Pacinian corpuscles) rapidly adapting mechanoreceptors was studied in parallel.
Results: The first evidence of Merkel cells showing the typical morphology and placement was at 13 weeks of estimated gestation age, and at this time positive immunoreactivity for PIEZO2 was achieved. PIEZO2 expression in axons terminals started at 23 WEGA in Pacinian corpuscles and at 36 WEGA in the case of Meissner corpuscles. The occurrence of PIEZO2 in Merkel cells, Meissner and Pacinian corpuscles was maintained for all the time investigated. Interestingly PIEZO2 was absent in most Aβ type I slowly adapting low-threshold mechanoreceptors that innervate MC while it was regularly present in most Aβ type I and type II rapidly adapting low-threshold mechanoreceptors that supplies Meissner and Pacinian corpuscles.
Conclusion: The present results provide evidence that human cutaneous mechanoreceptors could perform mechanotransduction already during embryonic development.

1. Introduction

Merkel cells (MC) are a subpopulation of epithelial cells (Morrison et al., 2009; Van Keymeulen et al., 2009) present in the basal stratum of the epidermis of most vertebrates (Moll et al., 2005; Boulais and Misery, 2007; Abraham and Mathew, 2019). They are especially abundant in touch-sensitive areas of human skin (Lacour et al., 1991; Boot et al., 1992). Most MC establish synaptic-like contacts with Aβ afferents at the dermo-epidermal junction (Halata et al., 2003a; b), and these MC-axon terminal complexes (also called MC-neurite complexes; MC-NC) work as slowly adapting type I low-threshold mechanoreceptors (SAI-LTMRs). MC-NC mediate gentle touch involved in tactile discrimination of shapes and textures (Johnson, 2001; Maricich et al., 2009; Abraira and Ginty, 2013; Maksimovic et al., 2014; Zimmerman et al., 2014). Thus, MC can be regarded as presynaptic cells (Halata et al., 2003a) that contain all components of the presynaptic machinery (active-zone molecules,

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[2] Both authors share Senior Authorship

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synaptic vesicle proteins) and release neurotransmitters (Haeberle et al., 2004; Maksimovic et al., 2013; Zimmerman et al., 2014).

Recently, direct evidence has pointed out that MCs are touch-sensitive and essential components of touch receptors in skin, since they transduce mechanical stimuli into electrical signals through the mechanoprotein PIEZO2 and consequently, induce action potentials in SAI-LTMRs (Ikeda et al., 2014; Maksimovic et al., 2014; Woo et al., 2014). PIEZO2 is a mechanically gated multipass transmembrane protein forming part of an ion channel required for mechanotransduction (Coste et al., 2010; Ranade et al., 2014; Honoré et al., 2015).

Pre- and post-natal changes in the morphology and immunohistochemical profile of human cutaneous Meissner and Pacinian corpuscles, which work as rapidly adapting type I (RAI-) and type II (RAII-) LTMRs respectively, have been recently reported in detail (Feito et al., 2018; García-Piqueras et al., 2019), but nevertheless, MC were not investigated in those researchers. Moll et al., (1984, 1986, 1992) studied the appearance and age-related changes in human cutaneous MC density by using immunohistochemistry for cytokeratins (CKs). Much later, Jenkins et al. (2019) analysed the conversion of basal keratinocytes into MC, innervation patterns and signalling pathways required to act as mechanoreceptors. However, when human MC start to express PIEZO2 during pre-natal development has never been considered.

Therefore, we designed the present study to investigate expression of CK20 and synaptic proteins (synaptophysin -Syn- and chromogranin A -ChrA) in MC during prenatal and postnatal (until 20 years-old) development. Furthermore, we studied the innervation timing of MC and especially, that of PIEZO2 expression, regarded as evidence of mechanotransduction. On the other hand, to complete our previous study (Feito et al., 2018), we analysed parallelly foetal and post-natal (until 20 years-old) timetable for PIEZO2 expression in Meissner and Pacinian corpuscles. Overall, the study aimed to better understand the sense of touch in development.

2. 2. Material and methods

2.1. Material and tissue treatment

Skin samples were obtained from the palmar aspect of the distal phalanx of fingers obtained during autopsy of foetuses (n = 18) and children deceased around birth (n = 5; age range 0–22 days) and from no known neurological diseased children and young adults (n = 26; age range 3 months-20 years-old) suffering partial or total finger amputation. Thus, the sampled age range was from 12 weeks of estimated gestational age (WEGA) to 20 years-old (Table 1). The used material in the present study belongs to the histological collection of the SINPOS Research Group at the University of Oviedo (Registro Nacional de Biobancos, Sección colecciones, Ref. C-0001627, responsible OG-S). It was obtained from the Departments of Pathology of Hospital Universitario Central de Asturias (Oviedo, Spain), Hospital Universitario Donostia (San Sebastián, Spain), Hospital José Molina Orosa (Lanzarote, Spain) and Complejo Hospitalario Universitario de Salamanca (Salamanca, Spain). Specimens were fixed in 10% formaldehyde with 1 M phosphate buffered saline (pH 7.6) for 24–36 h and processed for routinely embedding in paraffin. Pieces were cut into 10-µm-thick sections perpendicularly to skin surface and mounted on gelatine-coated microscope slides. The study was approved by the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. CElm, PAst: Proyecto 266/18). All of these materials were obtained in compliance with the Spanish Law (RD 1301/2006; Ley 14/2007; DR 1716/2011; Orden ECC 1414/2013).

Table 1
Data on the case material. wega: weeks of estimated gestational age; d: day; m: month; y: year; nd: not done. L/R: left/right. M/F: male/female.

Nº cases	Age	Gender	L-R hand/finger
3	12–13 wega	nd	R/1–5
4	16–18 wega	nd	R/1
2	22 wega	M (1), F(1)	R and L/1–3
4	23 wega	M(2), F(2)	R/1–2–3
2	28–30 wega	M(2)	L/1–2
3	34–36 wega	M(1), F(2)	R/1–2
2	0 d	M	R/1
2	2 d	M	R/1–2
1	22 d	nd	R/1–2
1	3 m	F	R/1–2
3	5–8 m	M(2), nd	R/1–2
4	1–3 y	M(2), nd	R/1–2
5	5–8 y	M(3), F(2)	R/1–2 and L1
6	9–10 y	M(5), F(1)	R/1–2 and L2
4	12–15 y	M(3), F(1)	R/L 1–2 and L1/2
3	18–20 y	M(2), F(1)	R/1–2 and L/1–3

2.2. Immunohistochemistry

Deparaffinized and rehydrated sections were processed for immunohistochemistry using the EnVision antibody complex detection kit (Dako, Copenhagen, Denmark), following the supplier's instructions. Briefly, endogenous peroxidase activity was inhibited with 3% H_2O_2 for 15 min, and non-specific binding was blocked by 25% bovine serum albumin for 20 min. Sections were incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal antibodies anti-cytokeratin 20 (CK20; Dako, Glostrup, Denmark, reference IS777, purchased prediluted), anti-chromogranin A (ChrA; Dako, reference M0869, purchased prediluted), anti-synaptophysin (Syn; Dako; reference M7315, used diluted 1:200); anti-neuron specific enolase (NSE; clone BBS/NC/IV-H14, Thermo Fisher Scientific, Rockford, IL, USA, reference NB100–65648; used diluted 1:200) and rabbit anti-PIEZO2 (Sigma-Aldrich, Saint Louis, MS, USA, reference PA5–72976, amino acid sequence FEDEN-KAAVRIMAGDNVEICMNLDAASFSQHNP; used diluted 1:200). Subsequently, sections were incubated with anti-mouse or anti-rabbit IgG for 90 min at room temperature. Finally, slides were washed in buffer solution before visualizing immunoreaction with diaminobenzidine as a chromogen, washed again, rehydrated, and mounted with Entellan© (Merck, Dramstadt, Germany). To ascertain structural details, sections were counterstained with Mayer´s haematoxylin.

2.3. Double immunofluorescence

Sections were also processed for simultaneous detection of PIEZO2 with either specific axonal (NSE), either Schwann related cells (S100 protein) or Merkel cell (CK-20, ChrA, Syn) proteins (Hartschuh et al., 1989; Llombart et al., 2005; Vega et al., 2009; Fukuhara et al., 2016; Cobo et al., 2021; Carcaba et al., 2022). Non-specific binding was reduced by incubation with a solution of 25% calf bovine serum in tris buffer solution (TBS) for 30 min. Sections were incubated with a 1:1 v/v mixture of polyclonal antibody against PIEZO2 and monoclonal antibodies against NSE, CK-20, ChrA, or Syn in a humid chamber overnight at 4º C. After rinsing with TBS, sections were incubated for one hour with CFL488-conjugated bovine anti-rabbit IgG (diluted 1:200 in TBS; sc-362260, Santa Cruz Biotechnology, Heidelberg, Germany) and then, rinsed and incubated again for another hour with CyTM3-conjugated donkey anti-mouse antibody (diluted 1:100 in TBS; Jackson-ImmunoResearch, Baltimore, MD, USA). Both steps were performed in a dark humid chamber at room temperature. Sections were finally washed, and cell nuclei were stained with DAPI (10 ng/mL). Triple fluorescence was detected by using a Leica DMR-XA automatic fluorescence microscope (Microscopía fotónica y Proceso de imágen, Servicios científico-técnicos, Universidad de Oviedo) coupled with a Leica

Table 2
Density of Merkel cells stablished on the basis of immunoreactivity for age different markers used. w: week; wega: weeks of estimated gestational age; d: day; m: month; y: year; n.i.: cell type not identified.

Age	CK20 +	ChrA +	Syn +	NSE +	PIEZO2 +
12–13 wega	+ n.i.	+ n.i.	0.9 ± 0.01	+ n.i.	+ n.i.
16–18 wega	+ n.i.	1.2 ± 0.1	1.7 ± 0.3	+ n.i.	+ n.i
22–23 wega	5.3 ± 0.5	4.9 ± 1	5.1 ± 0.3	4.8 ± 0.9	3.1 ± 0.5
28–30 wega	39.8 ± 2.4	38.3 ± 1.6	32.2 ± 2.1	40.1 ± 2.7	25.8 ± 3.1
34–36 wega	56.9 ± 2.2	51.9 ± 1.7	49.9 ± 2.3	52.9 ± 2.8	26.2 ± 1.2
0 d	32.6 ± 0.8	28.7 ± 1.2	32.1 ± 0.6	31.0 ± 1.2	11.8 ± 0.8
3 w	225.4 ± 3,5	216.8 ± 2.9	222.7 ± 2.8	206.9 ± 4.1	54.7 ± 3.5
3–8 m	30.3 ± 2.1	27.4 ± 1.9	30.0 ± 2.2	29.9 ± 2.3	16.9 ± 1.0
1–3 y	33.3 ± 3.1	27.9 ± 2.7	30.9 ± 2.1	31.6 ± 2.1	22.6 ± 1.3
5–10 y	30.6 ± 2.9	28.0 ± 1.9	29.8 ± 1.6	28.7 ± 1.8	27.3 ± 2.2
12–15 y	28.2 ± 0.9	28.1 ± 1	28.3 ± 1.2	26.6 ± 1.1	26 ± 1.9
18–20 y	32.7 ± 0.9	29.3 ± 1.5	31.9 ± 1.1	30.4 ± 1.3	30.8 ± 1.5

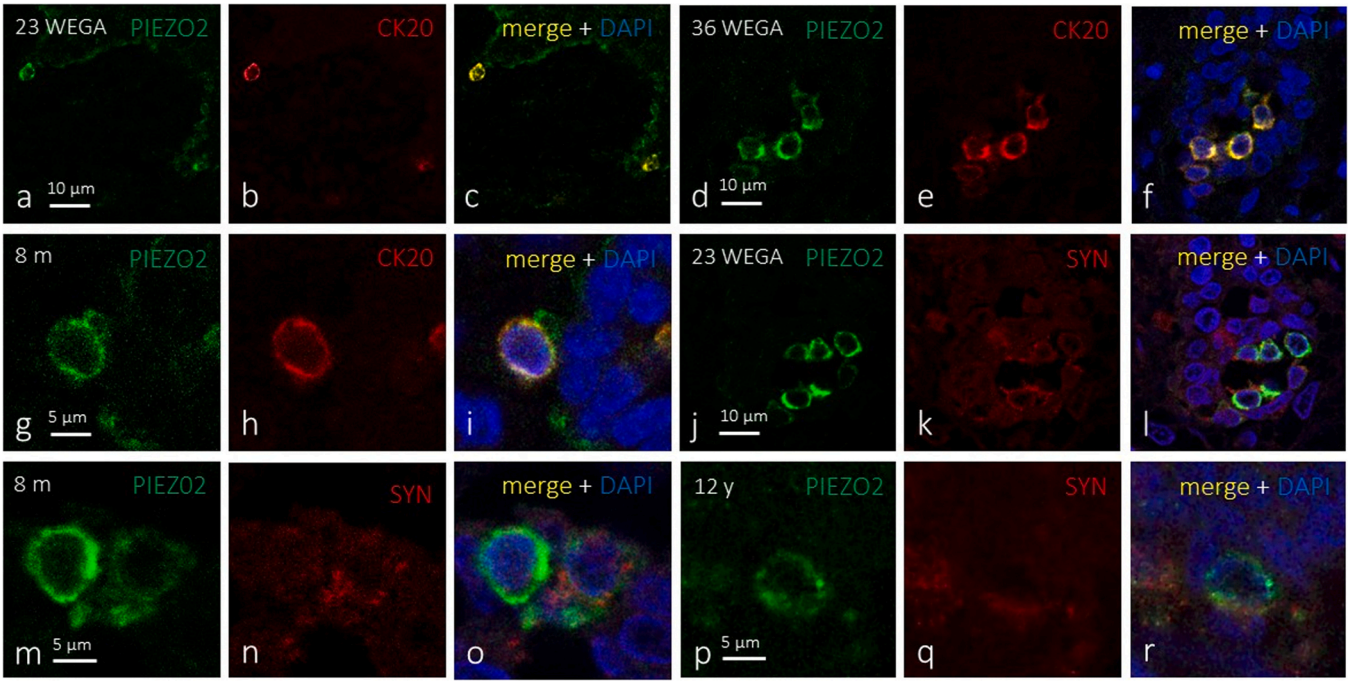

Fig. 1. Double immunofluorescence for PIEZO2 (green; **a, d, g, j, m, p**) and Cytokeratin 20 (CK, red; **b, e, h**) or Synaptophysin (SYN, red; **k, n, q**) in human glabrous skin during development. PIEZO2 totally co-localized with CK20 (yellow) in 23 wega (**c**), 36 wega (**f**) and 8 m (**i**), but did no co-localized with SYN (**l, n, r**). SYN was expressed in the synaptic pole (basal pole) of Merkel cells (**l, n, r**). In the first step of development, numerous clusters of Merkel cells were observed (**d, j**) and immunoreaction of PIEZO2 was positive in all of them. **a-f, j-l:** objective 40X/1.25 oil, pinhole 1.00, XY resolution 156 nm and Z resolution 334 nm. **g-i, m-r**: objective 63X/1.40 oil, pinhole 1.37, XY resolution 139.4 nm and Z resolution 235.8 nm.

Confocal Software (version 2.5; Leica Microsystems, Heidelberg GmbH, Germany), and captured images were processed using the software Image J (version 1.43 g; Master Biophotonics Facility, Mac Master University Ontario; www.macbiophotonics.ca, access on 11 January 2021).

For control purposes, representative sections were processed in the same way as described but either not using immune rabbit or mouse sera instead of primary antibodies or omitting primary antibodies when incubation. Furthermore, when available, additional controls were carried out using specifically preabsorbed antisera. Under these conditions, no positive immunostaining was observed (see Supplementary Material).

2.4. Quantitative study

Quantitative analyses were performed to determine densities of digital Merkel cells and sensory corpuscles during development and postnatal life. Number of Merkel cells, as well as Meissner and Pacini corpuscles, were calculated as follows: 4 fields x 5 sections per subject, 50 µm apart, were examined and quantified by microscopy at 10 × by two different observers, and obtained results were averaged. Data are expressed as mean ± sd/mm^2. Additionally, percentages of PIEZO2-positive Merkel cells and PIEZO2-positive sensory corpuscles were estimated, performing quantification with double immunofluorescence as described below: in regard to presumably functional Merkel cells, simultaneous detection of PIEZO2 and CK20 (as a specific marker) in five sections separated from each other by 50 µm; regarding PIEZO2-positive Meissner and Pacinian corpuscles, simultaneous detection of PIEZO2 and S100P in entire sections (for details see García-Piqueras et al., 2019; García-Mesa et al., 2022).

3. Results

3.1. Merkel cells display CK20 immunoreactivity from 22 to 23 WEGA

Immunoreactivity for the cytoskeletal intermediate filament CK20 was first detected in 13-WEGA skin samples and maintained all over the studied time (Fig1. 2b, e and h). However, based on CK20-

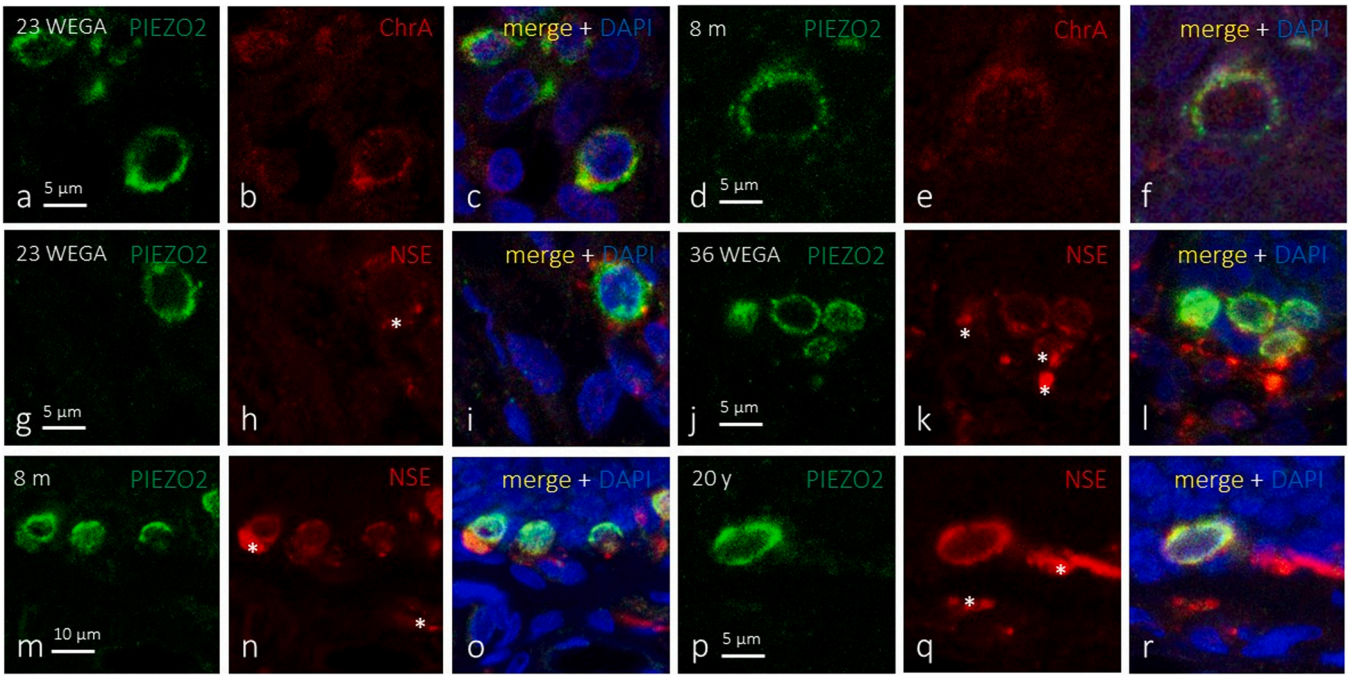

Fig. 2. - Double immunofluorescence for PIEZO2 (green; **a, d, g, j, m, p**) and Chromogranin A (ChrA, red; **b, e**) or Neural Specific Enolase (NSE, red; **h, k, n, q**) at different ages during development. ChrA immunoreaction pattern was diffuse and no-colocalized with PIEZO2 (**c, f**). In the case of the axonal marker NSE, in the 23 wega a diffuse pattern was observed around the positive PIEZO2 halo, corresponding to the Merkel cell (**i**), while in the last prenatal stages (**l**), as well as in the postnatal ones (**o, r**), the immunoreaction for NSE was found both in the Merkel cell and in the axon terminal that reaches the basal pole of the Merkel-neurite complex (asterisks in **h, k, n, q**). In spite of this, there is no co-localization between PIEZO2 and NSE (**i, l, o, r**). Objective 63X/1.40 oil, pinhole 1.37, XY resolution 139.4 nm and Z resolution 235.8 nm.

positive cell placement within epidermis as well as their morphology, they were identified as MCs just from 22 to 23. The immunolabelling pattern was similar in all cases, forming a peripheral ring-like halo. However, density of CK20-positive Merkel cells at different examined timepoints varied, being maximum at 3 postnatal weeks, and remaining rather stable from 3 to 8 months until 20 years-old (Table 2).

3.2. Merkel cells express Syn and ChrA from 22 to 23 WEGA

Syn and ChrA are proteins of presynaptic and secretory vesicles respectively, and both are present in mature human MCs. In the analysed digital skin samples, expression of Syn (Fig. 1k, n and q) and ChrA (Fig. 2b and e) began at 13 and 18 WEGA, respectively. Nevertheless, Merkel cells with typical location and morphology were observed from 23 WEGA onwards. Age-related variations in density of Syn-positive and ChrA-positive Merkel cells are shown in Table 2. Syn expression pattern was fine, granular, non-confluent and arranged in the most peripheral part of the cell; that of ChrA was also granular and formed a peripheral cytoplasmic ring.

3.3. Merkel cells start the expression of PIEZO2 at 22–23 WEGA

PIEZO2 is an essential mechanoprotein for the mechanotransduction process in MC-neurite complexes. Therefore, PIEZO2 occurrence within these cells can be regarded as indirect evidence of the acquisition of mechanosensitivity and mechanotransduction by these sensory complexes. The first evidence of PIEZO2 immunoreactivity in cells located at the basal stratum of epidermis was at 13 WEGA (Fig. 1a); however, just from 23 WEGA, the PIEZO2-positive cells display typical morphology and location of Merkel cells (Fig. 3). From that age and during all the analysed time, MC were PIEZO2-positive (Fig. 1a,d,g,j,m,p; Fig. 1a,d,g,j,m,p; Fig. 3), although their density experienced small variations depending on age (Table 2). Density of PIEZO2-positive MC was roughly equal to that of CK20-positive MC, but percentage of PIEZO2-positive cells increased from 3 weeks up to reach a maximum peak at 18–20 years-old (Table 3). At all ages, the immunolabelling pattern for PIEZO2 in MCs was granular, forming a peripheral cytoplasmic ring.

Another evaluated aspect in the present study was whether PIEZO2-immunoreactive cells are innervated. NSE was used as an axonal marker, although it also is expressed by MCs. This experiment allows us further to analyse whether axon terminals of LTMRs contacting MC express PIEZO2. Indeed, NSE was detected in MC cytoplasm from 13 WEGA to 20 years-old, showing a faint granular pattern at the periphery of the cytoplasm like a ring (Fig. 2h, k,n, and q). In addition, extracellular NSE-positive spots or axon profiles in apparent contact with MCs were detected since 13 WEGA. Occasionally, NSE-positive extracellular structures showed typical morphology of so-called Merkel tactile discs (Fig. 3n). It is important to note that as a rule, PIEZO2 was not detected in axon terminals of MC-neurite complexes (Fig. 3l, or and r).

Moreover, as expected, PIEZO2 co-localized completely or partially in MCs with all of the other investigated proteins (Figs. 1 and 2; Table 2).

3.4. Digital Meissner corpuscles display PIEZO2 immunoreactivity at the ending of the fetal period

In a recent work from our laboratory (Feito et al., 2018), we found that human digital Meissner corpuscles develop from the later prenatal stages. In agreement with those results, now we have observed that from 36 WEGA (Fig. 4a and b) and during the analysed time periods (Fig. 4c to e), axons of Meissner corpuscle are PIEZO2-positive, although the density changed slightly with age (Table 3).

3.5. Pacinian corpuscles display PIEZO2 early in development

Human digital Pacinian corpuscles acquire their typical morphology and basic structure from 24 WEGA (Feito et al., 2018). The

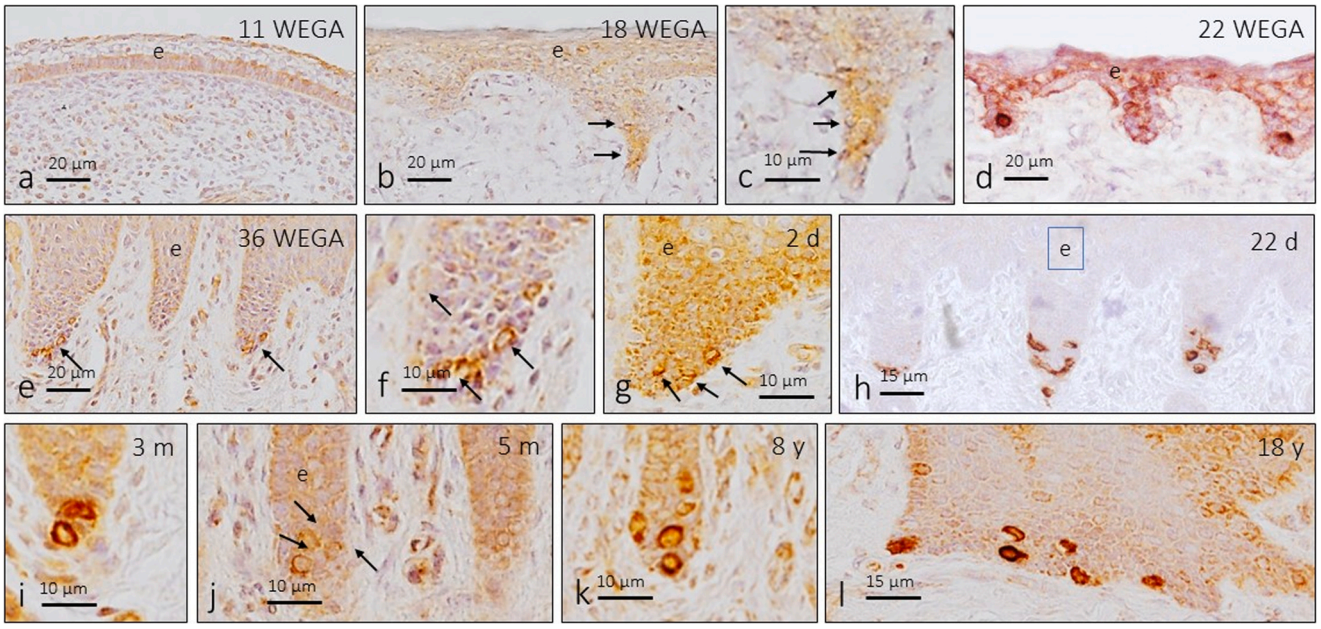

Fig. 3. Single immunohistochemistry for PIEZO2 during development. During the first trimester of development (see 11 wega), PIEZO2 was observed in epidermis (**a**). Merkel cells were first observed at 18 wega (arrows in **b,c**), with a light marking in the basal layer of epidermis, and the immunoreaction gets more intensive from 22 wega (**d**) and the number of Merkel cells increased at 36 wega (**e,f**), appearing clusters (arrows in **e** and **f**). In the postnatal stage, the number of this decreased, being similar during the adult stage (**g-l**), but its morphology change, losing their oval morphology in some cases from 18 years (**l**). e:epidermis.

Table 3
Percentage of Merkel cells, Meissner and Pacinian corpuscles displaying PIEZO2 immunoreactivity. Merkel cells displaying CK20 immunoreactivity were regarded as 100%, and the total number (100%) of Meissner and Pacinian corpuscles was determined in sections processed for detection of S100. n.d: not done; n.i: cell type not identified.

Age	Merkel cells % Merkel c. PIEZO 2 +	MC Corpuscle % MC PIEZO2 +	PC Corpuscles % PC PIEZO2 +
12–13 wega	n.i.	n.d.	n.d.
16–18 wega	n.i.	n.d.	n.d.
22–23 wega	58,49 ± 2.2	n.d.	38.2 ± 5.5
28–30 wega	62.8 ± 1.9	n.d	50.1 ± 4.7
34–36 wega	46.0 ± 1.6	16.3 ± 2.8	69.3 ± 4.7
0 d	36.2 ± 0.6	23.7 ± 2.9	76.3 ± 4.7
3 w	23.9 ± 1.1	22.3 ± 3.2	74.2 ± 3.9
3–8 m	55.7 ± 1.7	22.1 ± 3.1	72.8 ± 5.6
1–3 y	67.2 ± 2.1	33.6 ± 4.1	86.1 ± 5.2
5–10 y	89.1 ± 4.8	76.2 ± 7.3	98.8 ± 2.7
12–15 y	92,5 ± 2,2	98.2 ± 7.3	100
18–20 y	94,1 ± 1.6	96.4 ± 5.1	100

present findings show that the axon of Pacinian corpuscles become PIEZO2-positive at 23 WEGA and maintain its expression throughout all over the studied time (Fig. 4f-h). There were no significant age-related variations in percentage of PIEZO2-positive Pacinian corpuscles.

4. Discussion

We have previously described the temporal pattern as well as the proper immunohistochemical-profile acquisition of human digital Meissner and Pacinian corpuscles during development (Feito et al., 2018) and ageing (García-Piqueras et al., 2019). Now, we extend the research by the present study, analysing the prenatal development and postnatal age-related changes (until 20 years-old) in digital MCs, focusing on the time in which they start to display PIEZO2 immunoreactivity. In the analysis we also include the developmental expression of PIEZO2 by Meissner and Pacinian corpuscles. PIEZO2 occurrence in LTMRs and MCs can be regarded as indirect evidence of mechanosensing and mechanotransduction by these sensory structures because the attributed function to this ion channel (Woo et al., 2014).

The first step of our research was to establish from which moment MCs are present in human digital skin. MCs are differentiated epithelial-derived cells that account for less than 5% of the total cell population in epidermis (McGrath and Uitto, 2010; Oss-Ronen and Cohen, 2021) and are found in sensitive cutaneous and mucous areas (Hartschuh and Grube, 1979; Moll et al., 2005). They form sensory complexes with Aβ SA1-LTMRs to encode object features and gentle touch pressure (Johnson, 2001; Maksimovic et al., 2014; Maricich et al., 2012). MC express both epithelial and neuroendocrine markers. Their cytoskeletal intermediate filaments stain positively for low-molecular-weight cytokeratins like CK20 and their cytoplasm, for neuroendocrine proteins such as ChrA, protein gene product 9.5, NSE, and Syn (Halata et al., 2003; for a review see Abraham and Mathew, 2019).

In our hands, although CK20 elements were identified in epidermis from 13 WEGA, typical MCs were only detected from 22 to 23 WEGA. These results differ from those of Moll et al., (1984, 1986) and Moll and Moll (1992), who found CK20-positive MCs at 8 fetal weeks. Similar findings were obtained by Kim and Holbrook (1995), who found CK20-positive cells as early as 56 days of estimated gestational age (8 WEGA) in palmar epidermis. These discrepancies may be due to, at least in part, the anatomical skin sampling, the sample processing or the antibody sensitivity. Regarding the investigated neuroendocrine markers in MC (Hartschuh et al., 1989; Llombart et al., 2005; Fukuhara et al., 2016; Carcaba et al., 2022), they were detected in proper MCs at the same times of CK20 and following a parallel course during the investigated period of time.

As previously reported, we have found that density of MC varies throughout development. With the used method, density of MC in human digital skin increased from 22 to 23 WEGA until 3 postnatal weeks; thereafter, the number decreased abruptly and remained with no change until 20 years old. These results disagree with observations of Moll et al. (1984), Moll and Moll (1992), Boot et al. (1992) and Kim and Holbrook (1995), who found that MCs are considerably more numerous at early stages of development than in older foetuses. The reasons for these discrepancies must be

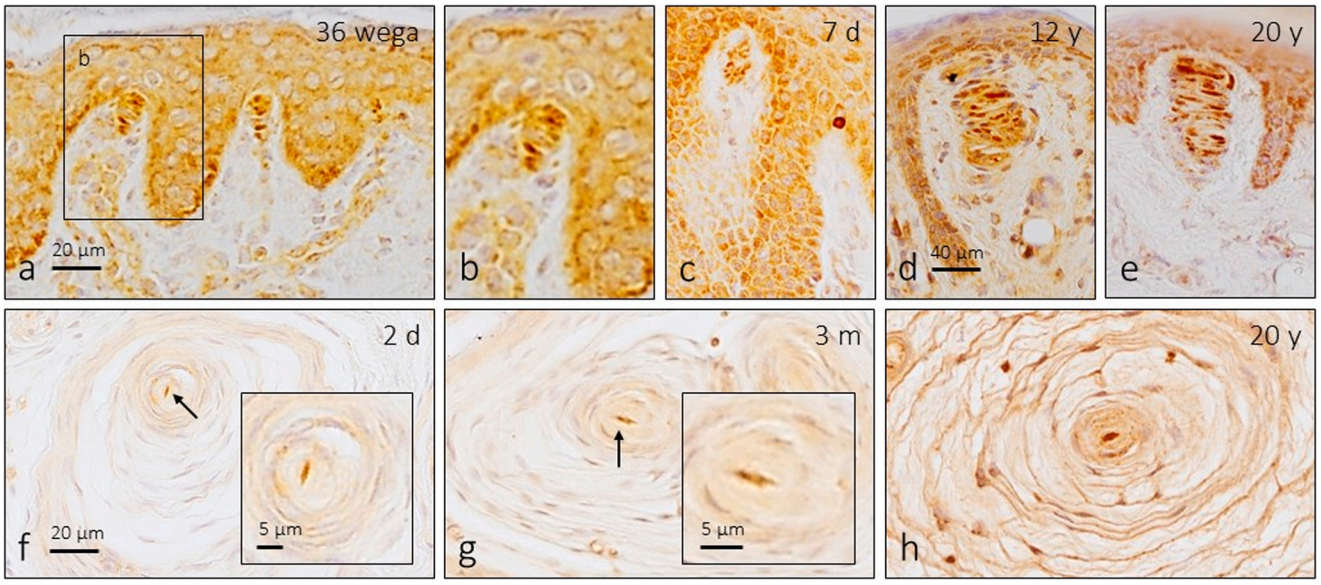

Fig. 4. Immunohistochemical detection of PIEZO2 in human digital skin focusing on Meissner and Pacinian corpuscles at different ages. PIEZO2 was observed from 36 wega in the axon of Meissner corpuscles (**a-e**), with a winding path between lamellar cells, which are slightly positive for PIEZO2 too. In contrast, Pacinian corpuscles were not positive for PIEZO2 until postnatal stage (see 2d; **f**), and the immunoreaction patterns was axonal (**f, g, h**).

reinvestigated, but the anatomical origin of skin samples and sensitivity of the used antibodies might account.

Most MC contact sensory afferents to form MC-neurite complexes in the basal epidermal layer. Therefore, we also investigated the specific time at which MC become innervate. The present results suggest that MC are innervated by the 22–23 WEGA. This time is considerably later than that reported by Narisawa and Hashimoto (1991), in which they recognized nerve-MC relationships in plantar skin of 12-week foetuses by using a combination of MC-specific antikeratin and neurofilament antibodies and showing that MC appearance preceded nerves. Nevertheless, some studies in mouse establish that the location and early differentiation of MCs are independent of nerves (Vielkind et al., 1995). Based on our experiments it is not possible to know if differentiated MCs predate nerves, since both were detected simultaneously at 22–23 WEGA.

Cutaneous MC in human skin displayed PIEZO2 immunoreactivity and density of PIEZO2-positive MC undergo age-dependent changes (García-Mesa et al., 2017; García-Piqueras et al., 2019). Now, we have observed that MC start to show PIEZO2-immunoreactivity earlier during development (22–23 WEGA), and it continues throughout the whole analysed period of time. Considering PIEZO2-positivity, MC density follows roughly a parallel trend to that described by MC when identified by expression of CK20 and other markers. However, not all CM were PIEZO2-positive. Percentage of PIEZO2-positive cells firstly decreased from 28 to 30 WEGA to three weeks and then increased progressively until 5–10 years-old. From this age to 20 years-old, approximately 92% of MC were PIEZO2-positive. In a previous study in older subjects (age range between 21 and 60 years-old), about 80% of MC were PIEZO2-positive (García-Mesa et al., 2017). Recently, Michel et al. (2021) have observed a decline in behavioural cutaneous touch sensitivity and PIEZO2-mediated mechanotransduction in cultured mouse dorsal root ganglia neurons during mouse maturation, but not thereafter.

As far as we know, this is the first study demonstrating PIEZO2 expression in prenatal MC, and the results suggest that MC are able to transduce mechanical stimuli during fetal life since PIEZO2 is an essential component in the mechanotransduction mechanism (Ikeda et al., 2014; Maksimovic et al., 2014; Woo et al., 2014). In support of this view, we observed that most PIEZO2-positive MC are in contact with nerve profiles, thus indicating they are innervated. Nevertheless, this indirect evidence needs to be confirmed by functional studies. In recent years, it has been confirmed a long-debated hypothesis, affirming that CM are mechanosensory cells required for tactile sensation: upon direct touch stimuli, MC display ionic currents mediated by PIEZO2, which induce neurotransmitter release, which in turn, triggers action potential firing of the Aβ SA1-LTMR (Ikeda et al., 2014; Maksimovic et al., 2014; Nakatani et al., 2015; Woo et al., 2014, 2015a; 2015b).

In the present study, we also analysed PIEZO2 expression in Meissner and Pacinian corpuscles from 11 WEGA to 20 years old. Our results are in complete agreement with previous studies from our lab (García-Mesa et al., 2017; García-P í queras et al., 2019), reporting PIEZO2 occurrence in axons of Meissner and Pacinian corpuscles, likewise as in Meissner-like corpuscles from murine skin (Ranade et al., 2014) and palatal mucosa (Moayedi et al., 2018).

There is an interesting result that emerges from our study: while almost all Aβ RAI- and RAII-LTMRs that supply Meissner and Pacinian corpuscles respectively, display PIEZO2 immunoreactivity, very few Aβ SAI-LTMRs that innervate MC are PIEZO2-positive. In contrast, accessory terminal cells of Meissner and Pacini corpuscles, i.e., the glial terminal cells, do not express PIEZO2 and those of the MC-neurite complexes, i.e., the MCs, do it. More studies are needed to explain these facts.

Touch is of capital importance for functional brain maturation (Nicolelis et al., 1996; Lauronen et al., 2006; Pihko et al., 2009; Nevalainen et al., 2014). Somatosensorial system provides information about touch, pressure, temperature, form, sharpness, hardness, undulation, etc. to the brain (Pleger and Villringer, 2013), and this information starts by the seventh gestational month, although the basic mechanisms are functional a little before (Pihko and Lauronen, 2004; Nevalainen et al., 2014). Cortical responses to touch at 2 years-old are similar as in adults (Lauronen et al., 2006; Pihko et al., 2009). In any case, touch plays key roles in the development of neurological (Nevalainen et al., 2014) and social (Croy et al., 2017) brain functions. The present results, together with our previous results (Feito et al., 2018), provide structural basis of the touch at the nervous system periphery. Whether these data can have clinical relevance remains to be determined in future studies. Tactile deprivation studies in hAlterations in somatosensory (touch and pain) behaviors are highly prevalent among people with autism spectrum disorders (ASDs).

However, the neural mechanisms underlying abnormal touch and pain-related behaviors in ASDs and how altered somatosensory reactivity might contribute to ASD pathogenesis has not been well studied. Here, we provide a brief review of somatosensory alterations observed in people with ASDs and recent evidence from animal models that implicates peripheral neurons as a locus of dysfunction for somatosensory abnormalities in ASDs. Lastly, we describe current efforts to understand how altered peripheral sensory neuron dysfunction may impact brain development and complex behaviors in ASD models, and whether targeting peripheral somatosensory neurons to improve their function might also improve related ASD phenotypes.uman, non-human primate, and rodents showed that early life experiences and developmental tactile stimulation are essential for proper brain development, cognition and adult social behaviours. Individuals with autism spectrum disorders display abnormalities in processing tactile modalities and these impairments correlate with deficits in social behaviour (Rogers and Ozonoff, 2005; Mikkelsen et al., 2018; Orefice, 2020). Adults with autism spectrum disorders have been shown to exhibit lower thresholds for tactile perception of vibro-tactile stimuli, suggesting a specific hypersensitivity in Aβ RAII-LTMRs pathways (Blakemore et al., 2006). In concordance with this finding, Cascio and colleagues found that adults with autism spectrum disorders display increased sensitivity to both vibration and thermal pain (see Cascio et al., 2016).

Ethical statement

This study was approved by the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. CElm, PAst: Proyecto 266/18).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aanat.2022.151953.

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3.3. Resultados – Publicación 3

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Merkel cells and Meissner's corpuscles in human digital skin display Piezo2 immunoreactivity

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Abstract

The transformation of mechanical energy into electrical signals is the first step in mechanotransduction in the peripheral sensory nervous system and relies on the presence of mechanically gated ion channels within specialized sensory organs called mechanoreceptors. Piezo2 is a vertebrate stretch-gated ion channel necessary for mechanosensitive channels in mammalian cells. Functionally, it is related to light touch, which has been detected in murine cutaneous Merkel cell–neurite complexes, Meissner-like corpuscles and lanceolate nerve endings. To the best of our knowledge, the occurrence of Piezo2 in human cutaneous mechanoreceptors has never been investigated. Here, we used simple and double immunohistochemistry to investigate the occurrence of Piezo2 in human digital glabrous skin. Piezo2 immunoreactivity was detected in approximately 80% of morphologically and immunohistochemically characterized (cytokeratin 20^+, chromogranin A^+ and synaptophisin$^+$) Merkel cells. Most of them were in close contact with Piezo2$^-$ nerve fibre profiles. Moreover, the axon, but not the lamellar cells, of Meissner's corpuscles was also Piezo2$^+$, but other mechanoreceptors, i.e. Pacinian or Ruffini's corpuscles, were devoid of immunoreactivity. Piezo2 was also observed in non-nervous tissue, especially the basal keratinocytes, endothelial cells and sweat glands. The present results demonstrate the occurrence of Piezo2 in cutaneous sensory nerve formations that functionally work as slowly adapting (Merkel cells) and rapidly adapting (Meissner's corpuscles) low-threshold mechanoreceptors and are related to fine and discriminative touch but not to vibration or hard touch. These data offer additional insight into the molecular basis of mechanosensing in humans.

Key words: cutaneous mechanoreceptors; mechanotransduction; Meissner's corpuscles; Merkel cells; Piezo2 ion channel.

Introduction

Mechanotransduction can be defined as the transformation of mechanical energy into biological signals, and in the peripheral sensory nervous system, it results in the generation of an electrical signal that is the first step in mechanosensing. It is currently accepted that mechanically activated ion channels initiate mechanosensing, from cutaneous light touch to muscular proprioception. Nevertheless, the molecular and neural mechanisms underlying this process and the precise identity of these molecules in vertebrates are still elusive. The ion channel mechanosensor candidates belong to the superfamilies of the Degenerin/Epithelial Na^+ channels (especially the family of acid-sensing ion channels, ASICs) and transient receptor potential (TRP) ion channels, the two-potassium pore (K_{2P}) channels family, and the proteins encoded by *Piezo1* and *Piezo2* genes (Lumpkin & Caterina, 2007; Lumpkin et al. 2010; Roudaut et al. 2012; Delmas & Coste, 2013; Ranade et al. 2015; Sharif-Naeini, 2015).

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Piezo2 is a vertebrate stretch-gated multipass transmembrane protein necessary for non-selective cationic mechanosensitive channels in mammalian cells (Coste et al. 2010). Functionally, it is related to light, but not harsh, mechanical touch. In the peripheral nervous system, Piezo2, at the mRNA or protein level, has been detected in sensory neurons (Coste et al. 2010; Bron et al. 2014; Ranade et al. 2014; Alamri et al. 2015) in slowly adapting (SA) low-threshold mechanoreceptors (LTMRs), Merkel discs (consisting of Merkel cells and Aβ-afferent nerve endings) and isolated Merke cells (Ikeda et al. 2014; Maksimovic et al. 2014; Ranade et al. 2014; Woo et al. 2014, 2015a); in the rapidly adapting (RA) LTMRs, Meissner's corpuscles and lanceolate nerve endings (Ranade et al. 2014); and in muscle spindles (Woo et al. 2015b). Consistent with its role, Piezo2-deficient animals show an almost complete deficit in light-touch sensation and proprioception without affecting other somatosensory functions (Ranade et al. 2014; Woo et al. 2015a). *Piezo2* mutations in humans result in a selective loss of touch perception and a decrease in proprioception (Chesler et al. 2016; Mahmud et al. 2016). In addition to these sensory deficits, mutations in *Piezo2* are responsible for complex syndromes that involve joints, ocular muscles and bones (see McMillin et al. 2014; Alisch et al. 2016; Chesler et al. 2016).

The human glabrous skin contains sensory structures known collectively as mechanoreceptors, where mechanotransduction occurs (McGlone & Reilly, 2010; Roudaut et al. 2012; Fleming & Luo, 2013; Hao et al. 2015). They show different morphologies, such as Meissner's corpuscles, Pacinian corpuscles, Ruffini's corpuscles, and Merkel cell–neurite complexes (McGlone & Reilly, 2010; Zimmerman et al. 2014) but are similar in structure and immunohistochemical properties (Vega et al. 2009) and functionally work as SA- and RA-LTMRs (see Olson et al. 2016).

Based on actual knowledge of the molecular basis of mechanotransduction, it can be hypothesized that the ability of mechanoreceptors to detect mechanical stimuli relies on the presence of mechanosensitive ion channels within them and subsequently on the activation of the peripheral branch of LTMR sensory neurons (Abraira & Ginty, 2013). Previous studies in humans have reported the occurrence of the putative mechanosensing ion channels ASICs, TRPC6 and TRPV4 in Meissner's corpuscles, Pacinian corpuscles and Merkel cell–neurite complexes (Calavia et al. 2010; Cabo et al. 2012, 2015; Alonso-González et al. 2017). However, as opposed to Piezo2, those ion channels have not been shown to have mechanotransducer properties in vertebrates (see Delmas & Coste, 2013; Ranade et al. 2015).

To our knowledge, the distribution of Piezo2 in human skin has never been investigated. Here, we used immunohistochemistry to investigate the presence of Piezo2 in digital skin mechanoreceptors. This study aimed to better understand the molecular basis of mechanosensing in humans.

Materials and methods

Materials and treatment of tissues

Skin samples were obtained from the palmar aspect of the distal phalanx of amputated hand fingers (n = 12) from subjects free of neurological disease (age range 21–60 years; six females and six males). The material was collected within 3 h after amputation and was obtained in the Department of Plastic Surgery of the Hospital Universitario Central de Asturias (HUCA), Oviedo, Spain. The specimens were fixed in 4% formaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 24 h, dehydrated and routinely embedded in paraffin. All materials used in the present study were obtained in compliance with Spanish Laws and according to the guidelines of the Declaration of Helsinki II (World Medical Association, 2013).

Single immunohistochemistry

Deparaffinized and rehydrated sections were processed for detection of Piezo2 using the EnVision antibody complex detection kit (Dako, Copenhagen, Denmark) following the supplier's instructions. Briefly, endogenous peroxidase activity was inhibited (3% H_2O_2 for 15 min), and non-specific binding was blocked (10% bovine serum albumin for 20 min). Sections were then incubated overnight at 4 °C with the primary antibody. The antibody against Piezo2 was polyclonal raised in rabbit (Sigma-Aldrich, Madrid, Spain), and recognizes the following amino acid sequence: FEDENKAAVRIMAGDNVEICMNLDAASFSQHNP (manufacturer's notice); it was used diluted to 1 : 200. Subsequently, the sections were incubated with anti-rabbit EnVision system-labelled polymer (Dako-Cytomation) for 30 min. Finally, the slides were washed with buffer solution, and the immunoreaction was visualized with diaminobenzidine as a chromogen, washed, dehydrated, and mounted with Entellan® (Merck, Dramstadt, Germany). To ascertain structural details, the sections were counterstained with Mayer's haematoxylin.

Double immunofluorescence

Sections were also processed for simultaneous detection of Piezo2 together with specific markers for Schwann cells (S100 protein) or axons [200 kDa neurofilaments (NFP) and neuron-specific enolase (NSE); Vega et al. 2009] and for Merkel cells [chromogranin (ChrA), synaptophysin (Syn) and cytokeratin 20 (CK20); Llombart et al. 2005; Fukuhara et al. 2016]. Non-specific binding was reduced by incubating the sections for 30 min with a solution of 25% calf bovine serum in Tris buffer solution (TBS). The sections were incubated overnight at 4 °C in a humid chamber with a 1 : 1 v/v mixture of the polyclonal antibody against Piezo2 described above (used diluted 1 : 100) with monoclonal antibodies against S100 protein [GeneTex, S100 (4C4.9), GTX24066, used diluted 1 : 2000), NFP (Santa Cruz Biotechnology, CA, USA; NF-H (RNF402), sc-32729, used diluted 1 : 1000), NSE (Dako, Glostrup, Denmark; BBS/NC/VI-H14, used diluted 1 : 1000), Syn [Dako, (SY38), IR776, prediluted], CK20 (Dako, (K_s20.8-, IS777, prediluted), and chromogranin A [Boehringer Mannheim, Mannheim, Germany; LK2H10(9), 1199 021, used

diluted 1 : 200]. After rinsing with TBS, the sections were incubated for 1 h with CFL488-conjugated bovine anti-rabbit IgG (sc-362260, Santa Cruz Biotechnology), diluted to 1 : 200 in TBS, then rinsed again and incubated for another hour with Cy™3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch, Baltimore, MD, USA) diluted to 1 : 100 in TBS. Both steps were performed at room temperature in a dark, humid chamber. Sections were finally washed and the cell nuclei stained with DAPI (10 ng mL^{-1}). Triple fluorescence was detected using a Leica DMR-XA automatic fluorescence microscope (Microscopía fotónica y proceso de imágen, servicios científico-técnicos, Universidad de Oviedo) coupled with Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany), and the images captured were processed using ImageJ software version 1.43g [Master Biophotonics Facility, McMaster University Ontario (www.macbiophotonics.ca)].

For control purposes, representative sections were processed in the same way as described but using non-immune rabbit or mouse sera instead of the primary antibodies or omitting the primary antibodies in the incubation. Furthermore, when available, additional controls were carried out using specifically preabsorbed antisera. Under these conditions, no positive immunostainning was observed (data not shown).

Quantitative study

The percentage of Merkel cells and Meissner's corpuscles showing immunoreactivity for Piezo2 was calculated in five sections, 100 μm apart from each specimen, and processed for simultaneous detection of Piezo2-CK20 and Piezo2-S100 protein. The counts were made by two independent observers. Images were captured with a Leica SCN400 Scan coupled with a Nikon Eclipse 80i microscope and a Nikon DS-Ri1 camera, and they were viewed with the SlidePath Gateway LAN program (Leica). The results are expressed as the means ± standard deviations of the percentages of each category of immunoreactive Merkel cells and Meissner's corpuscles.

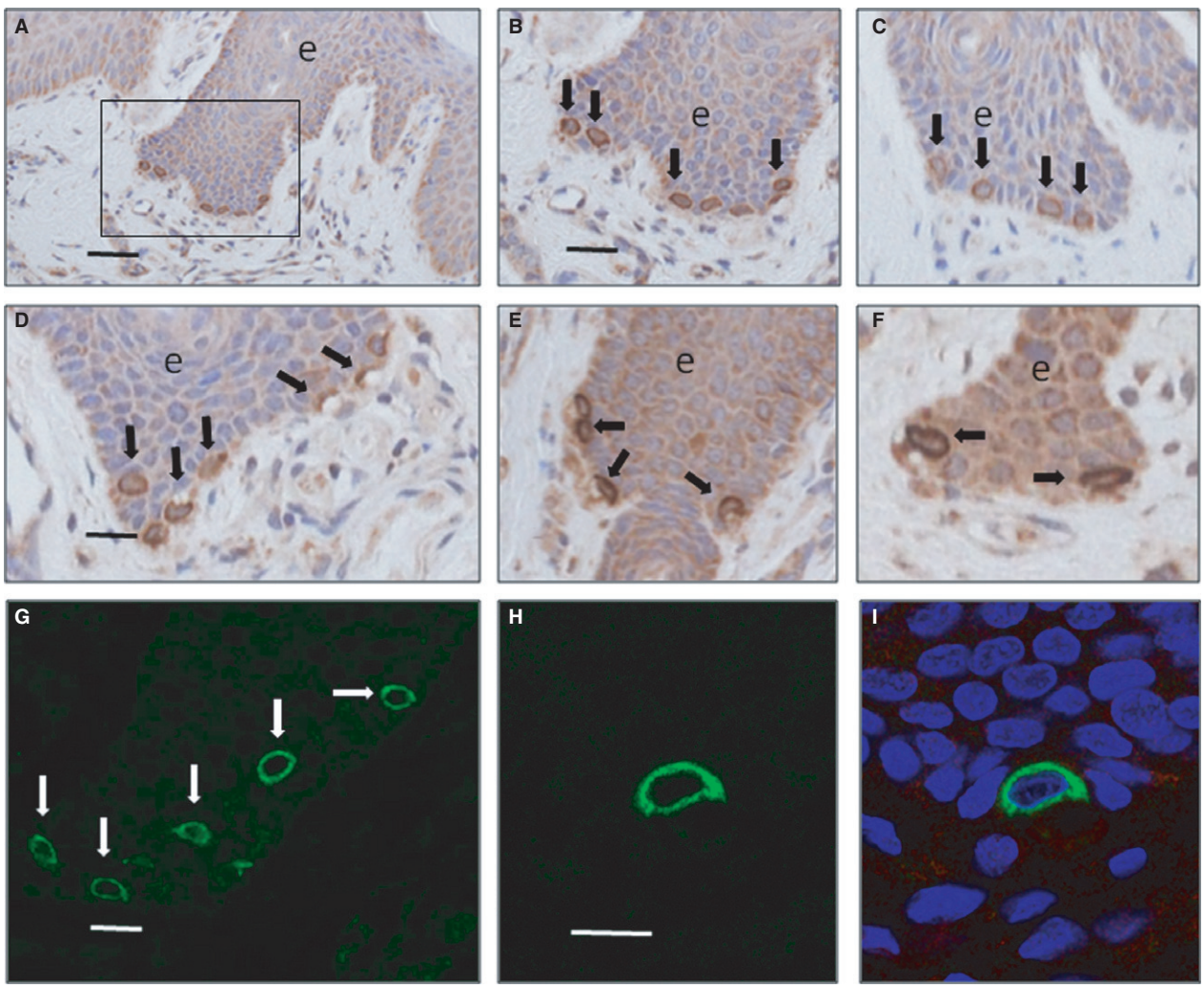

Fig. 1 Immunoreaction positive for Piezo2 in human digital Merkel cells. Single immunohistochemistry (A–F) or single immunofluorescence (G–I) was used to localize Piezo2 in human digital Merkel cells. The immunostaining showed a peripheral cytoplasmic halo (A–I). The Merkel cells were found isolated or forming clusters (arrows in B–G). Scale bar: 50 μm (A), 40 μm (B,C), 20 μm (D–F), 50 μm (H). (G) Objective 40×/1.25 oil; pinhole airy (AU) 1.95, XY resolution 156 nm and Z resolution 323 nm. Scale bar: 10 μm. (H–I) Objective 63×/1.25 oil; pinhole AU 1.55, XY resolution 156 nm and Z resolution 366 nm. Scale bar: 20 μm. e, epidermis.

Results

Merkel cells display Piezo2 immunoreactivity

Among the basal layer of the epidermal papillary ridges, abundant cells showing a peripheral cytoplasmic halo displayed strong immunoreactivity for Piezo2 and were occasionally found isolated but more often forming clusters (Fig. 1). Based on their morphology and location within the epidermis, these cells were identified as Merkel cells. Nevertheless, not all cells showing morphological features of Merkel cells were Piezo2-positive (Fig. 2). To identify definitively the epidermic Piezo2-positive cells, double immunohistochemistry was carried out using markers for

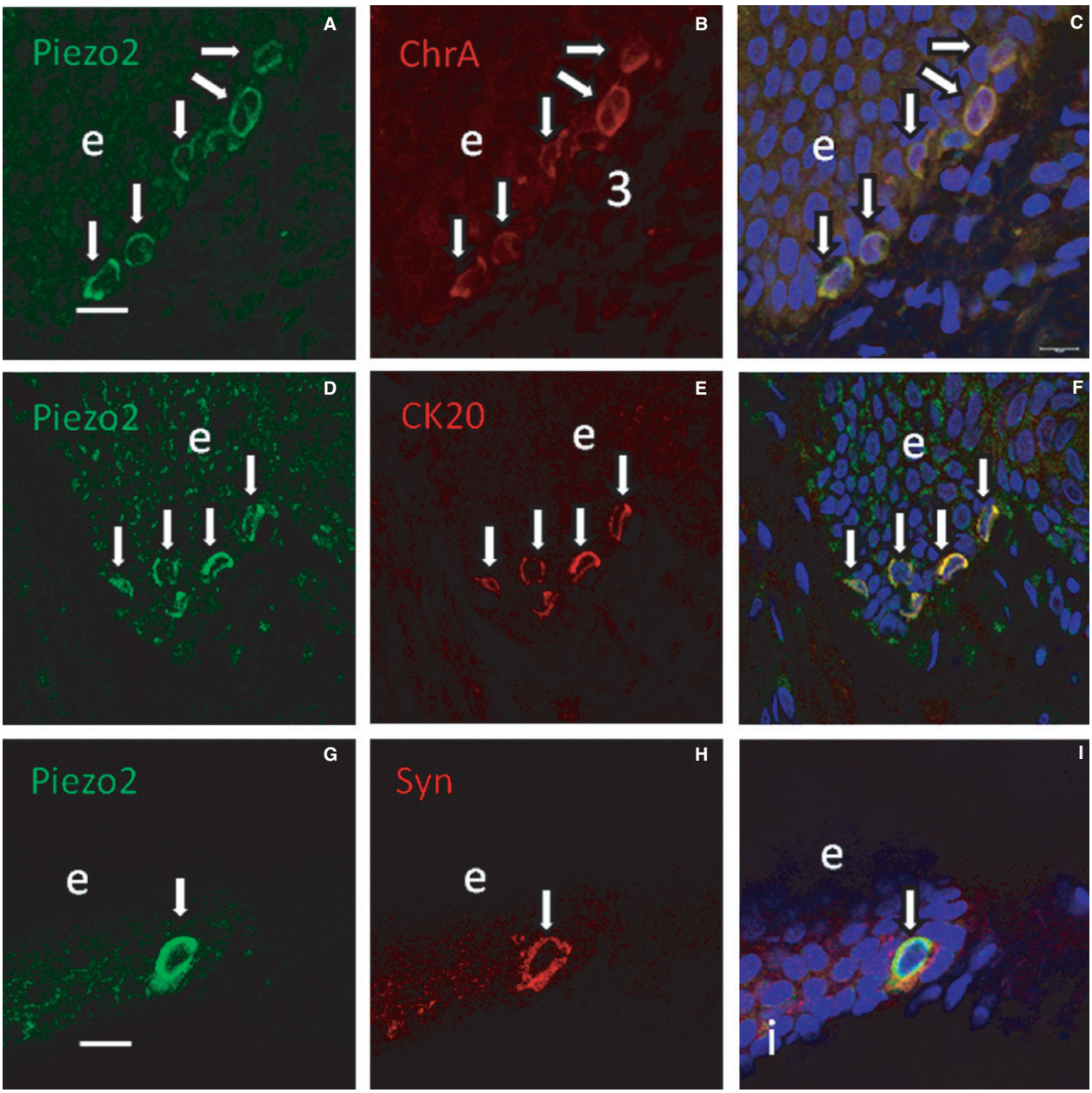

Fig. 2 Merkel cells are not all Piezo2-positive. Piezo2 was detected in most human Merkel cells using single immunohistochemistry. Cells with morphology and placement as Merkel cells are identified by an arrow; piezo2-positive (black arrow) and piezo2-negative (red arrow) (A–C). Human digital Merkel cells are labelled with anti-NSE antibody conjugated with CyTM3 (red fluorescence) and anti-Piezo2 conjugated with Alexa fluor 488 (green fluorescence). There was no co-localization of NSE and Piezo2 in the same cells (D–F). None of the cells showing morphological or immunohistochemical features of Merkel cells was Piezo2-positive; approximately 28 ± 4.3% lacked immunoreactivity for this protein. Scale bar: 10 μm (A–C). (D–F) Objective 40×/1.25 oil; pinhole AU 1.95, XY resolution 156 nm and Z resolution 323 nm. Scale bar: 10 μm. e, epidermis.

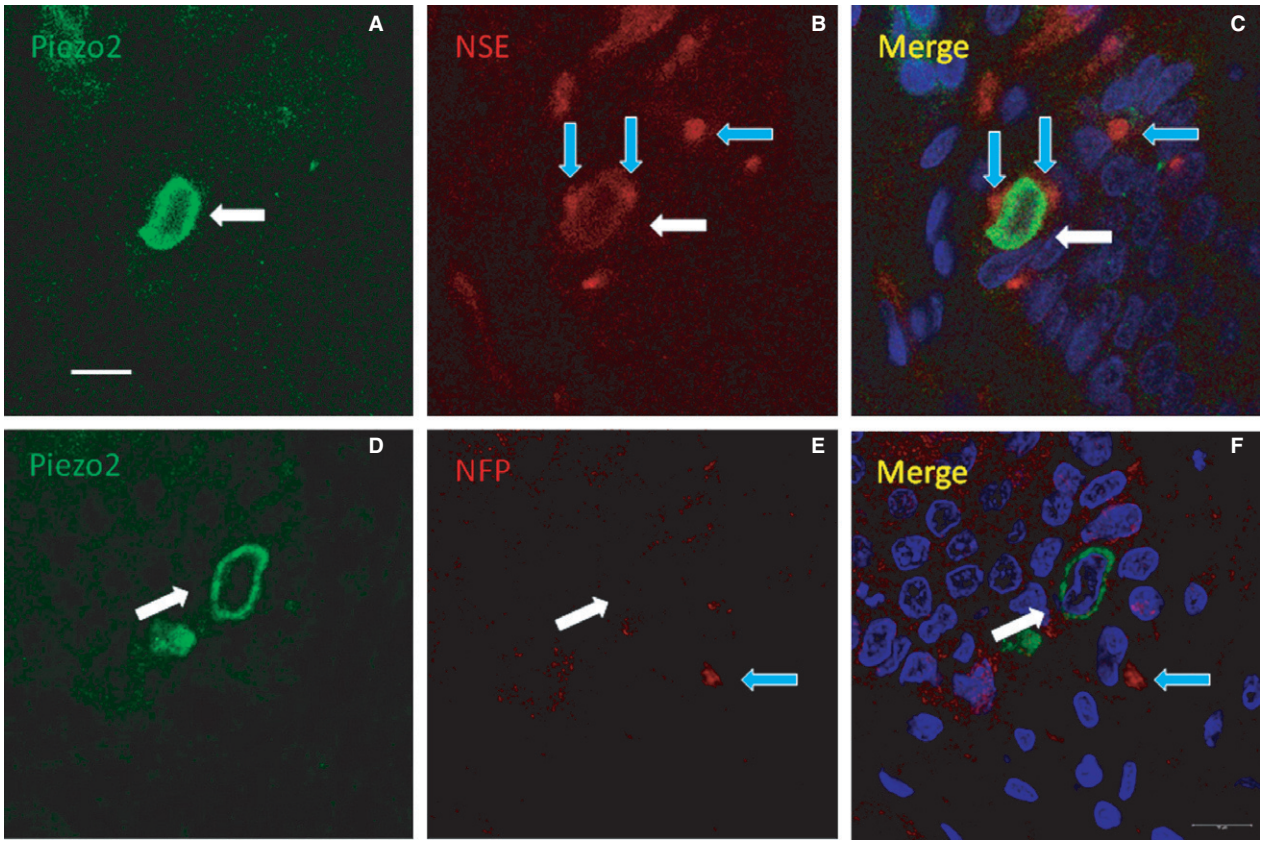

Fig. 3 Immunohistochemical characterization of Piezo2-positive cells in humans. Localization of Piezo2 immunoreactivity (A,D,E, green fluorescence) and ChrA, CK20 and Syn (B,E,H, red fluorescence, respectively) in human digital Merkel cells. When the images overlapped, there was co-localization of Piezo2-ChrA (C) and Piezo2-CK20 (F). Piezo2 and Syn did not co-localize, although immunostaining appeared within the same cells (E) (arrow indicates the Merkel cells). (A–F) Objective 40×/1.25 oil; pinhole AU 1.95, XY resolution 156 nm and Z resolution 366 nm. Scale bar: 10 μm. (G–I) Objective 63×/1.40 oil; pinhole AU 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm. Scale bar: 20 μm. e, epidermis.

Merkel cells together with Piezo2. Merkel cells displayed immunoreactivity for ChrA (Fig. 2a–c), CK20 (Fig. 2d–f) and Syn (Fig. 2g–i). In some cells, Piezo2 clearly co-localized with these Merkel cell markers, whereas in others (as was the case for Syn), both proteins were independent but localized within the same cells. In addition, the occurrence of NSE immunoreactivity in Merkel cells (Masuda et al. 1986; Isgrò et al. 2015) was detected in both Piezo2-negative (Fig. 3e) and Piezo2-positive (Fig. 4b) Merkel cells. Considering the count of CK20-positive cells as the total population of Merkel cells, approximately 21 ± 4.3% of them lacked Piezo2 immunoreactivity. On the other hand, a variable population of Merkel cells was intimately associated with a nerve terminal, but some were not. To establish whether Piezo2-positive Merkel cells were innervated, double immunolabelling for Piezo2 and NFP, NSE and S100 protein was performed. Most Piezo2-positive cells were in contact with NSE (Fig. 4a–c) or NFP (Fig. 4d–f) nerve profiles, but 16.4 ± 3.9% were apparently without nerve contact. Nerve fibres displaying S100 protein immunoreactivity were never found within the epidermis and were therefore considered to be in contact with Merkel cells (data not shown).

Piezo2 immunostaining in the axon of Meissner's corpuscles

We also explored Piezo2 immunoreactivity in Meissner's corpuscles. Using single immunohistochemistry, all Meissner's corpuscles examined showed a pattern of immunostaining consistent with its localization in the axons. Nevertheless, differences in the corpuscular Piezo2 profiles were observed, as indicated by the tortuous trajectory of the axon and the plainness of the section (Fig. 5). To confirm the axonic localization of Piezo2, double immunostaining was performed to label the axon with NFP and NSE and the lamellar cells with S100 protein. Piezo2 was co-localized with axonic markers labelling the whole axon profile (Fig. 6) or only axon segments (Fig. 7). When studied at high magnification in transverse sections of the axon, it was observed that Piezo2 was localized at the periphery, presumably in the axolemma (Fig. 7). As Piezo2 was not detected co-localized with S100 protein, it can be assumed that lamellar cells lack Piezo2 (Fig. 8). However, occasionally, a faint, diffuse and granular Piezo2 immunostaining was observed in these cells.

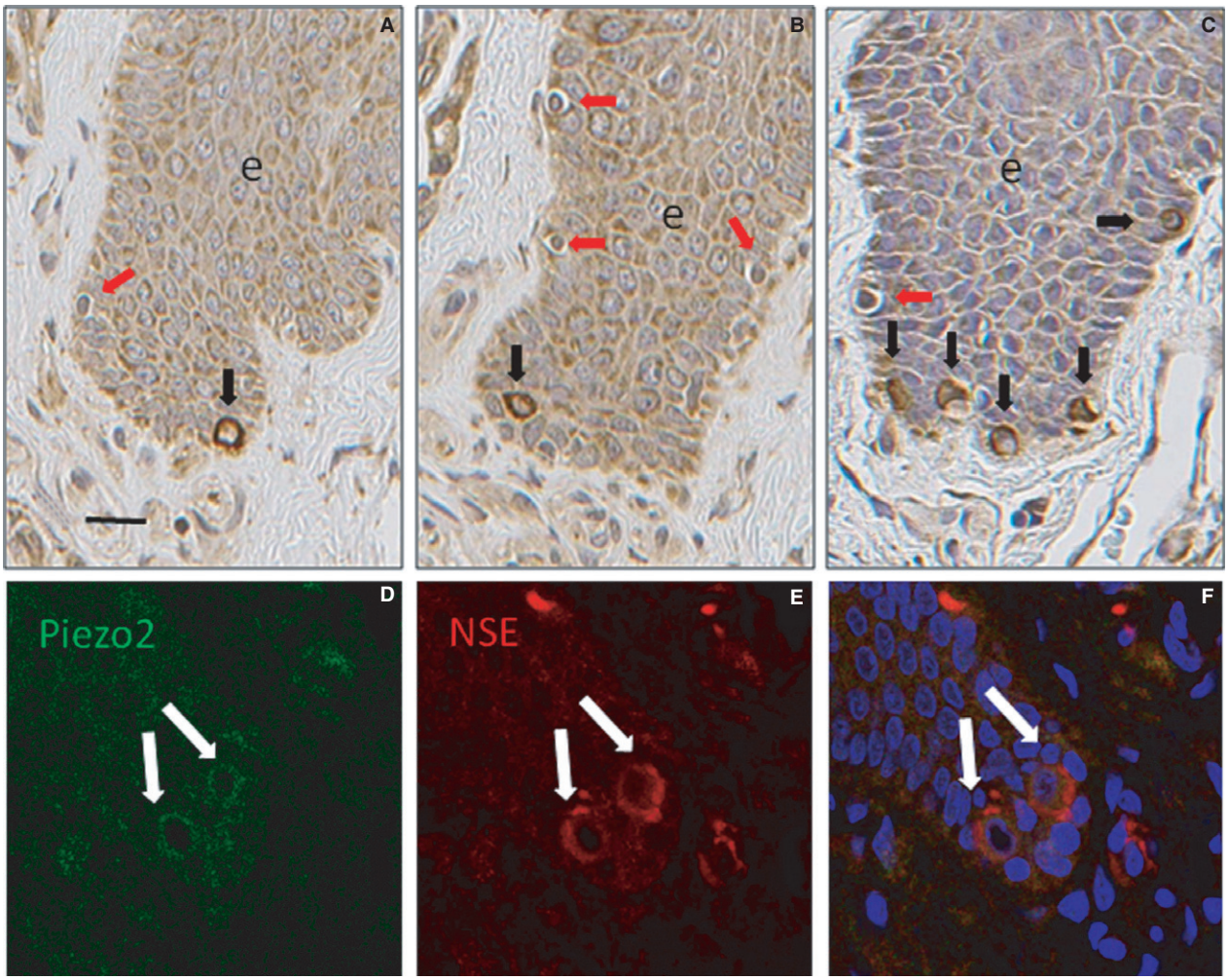

Fig. 4 Relation between Piezo2 Merkel cells and nerve terminal. Double immunofluorescence with Piezo2 (A,D, green fluorescence) identified Merkel cells (white arrow), and specific monoclonal antibodies, axonic markers NSE and NF (B,E, red fluorescence), marked terminal nerves (blue arrow). Some Merkel cells are intimately associated with a nerve terminal (C) but some are not (F). (A–F) Objective 63×/1.40 oil; pinhole AU 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm. Scale bar: 10 μm.

Other morphotypes of sensory corpuscles and cutaneous nerves

No specific immunoreactivity for Piezo2 was detected in other kinds of cutaneous mechanoreceptors, such as Pacinian (Fig. 9a,d) or Ruffini corpuscles (data not shown). In the superficial and deep dermal nerves, axons displaying Piezo2 were observed (Fig. 9a).

Piezo2 immunoreactivity in non-nervous cutaneous tissue

In addition to Merkel cells and Meissner's corpuscles, Piezo2 immunoreactivity was detected in several cutaneous tissues. In the epidermal keratinocytes, especially the basal ones, there was occasionally immunoreactivity showing a faint cytoplasmic pattern (Fig. 9a,e). In the dermis, both the endothelial and muscular layers of blood vessels were also Piezo2-positive (Fig. 9e), as were the acini and ductal cells of the sweat glands (Fig. 9a–c).

Although no specific studies were carried out, gender or age-related differences were not evident in the density of Piezo2-positive Merkel cells or Meissner's corpuscles.

Discussion

The activation of channels that results in ion flow in response to mechanical stimuli initiates the conversion of mechanical stimuli into electrical signals as a part of the complex process termed mechanotransduction (Lumpkin & Caterina, 2007; Lumpkin et al. 2010; Delmas & Coste, 2013; Ranade et al. 2015). Currently, members of the TRP, ASIC2, K_{p2} and Piezo ion channel families are considered potential mechanosensitive channels (Liedtke, 2005; Gu & Gu, 2014; Ranade et al. 2015; Sharif-Naeini, 2015). However, it has not been fully established whether they are true

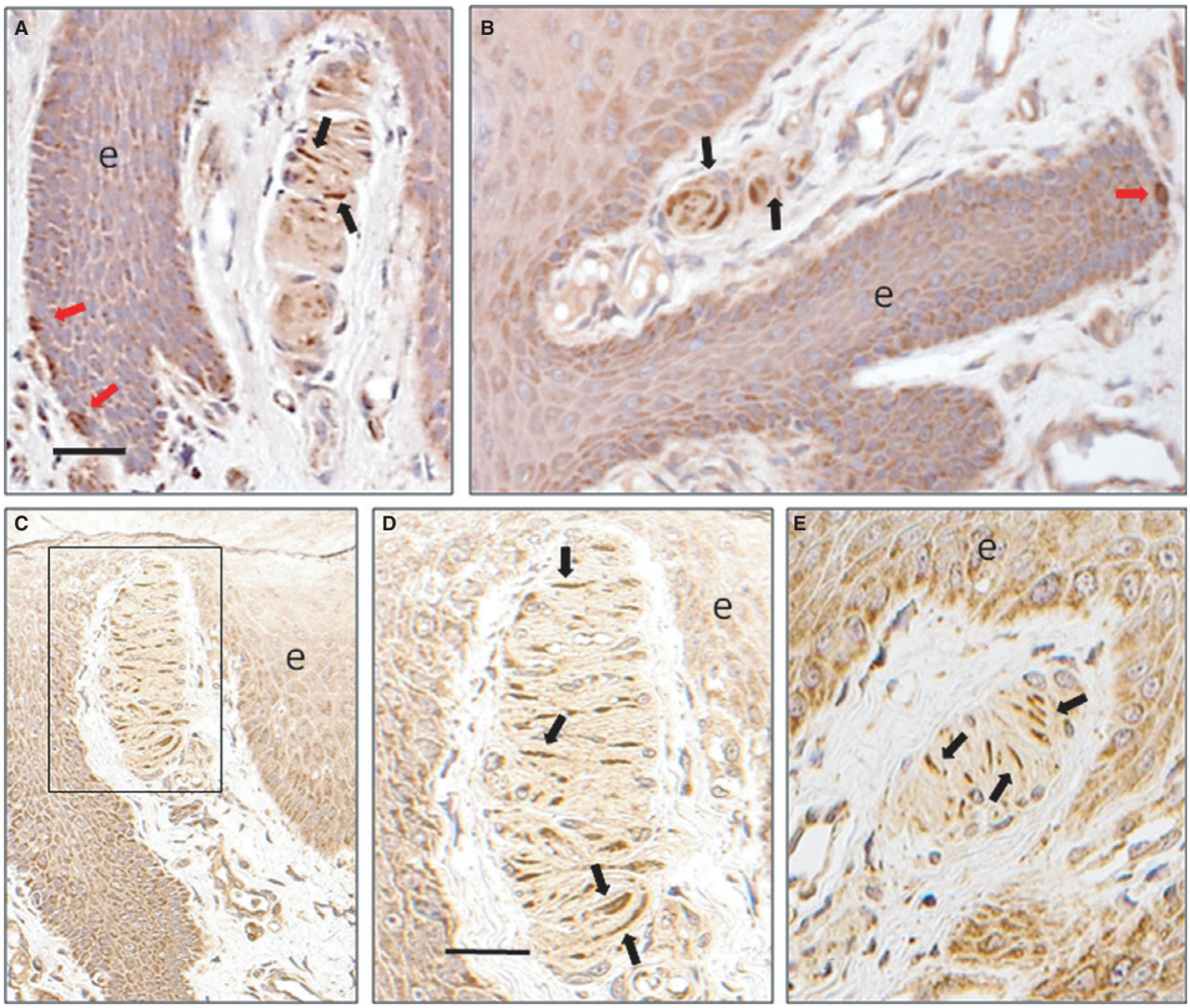

Fig. 5 Piezo2 in human digital Meissner's corpuscles. Localization of Piezo2 in human digital Meissner's corpuscles (A–E, black arrows) and Merkel cells (A–B, red arrows). The corpuscular Piezo2 profiles were detected along an axon flexuous trajectory. Scale bar: 40 μm (A–C), 20 μm (D–E). e, epidermis.

mechanotransducers or are only indirectly required for mechanotransduction, as there is no conclusive demonstration that most of these ion channels are mechanically gated. In addition, some of these mechanosensitive ion channels can be opened independently of mechanical forces (Honoré et al. 2015; Nakatani et al. 2015).

In vertebrates, mechanotransduction occurs in specialized sensory organs called mechanoreceptors (Vega et al. 2009; McGlone & Reilly, 2010) and involves the activation of the cutaneous branch of the axon of cutaneous LTMRs, a heterogeneous set of primary somatosensory neurons that function to sense mechanical forces (Roudaut et al. 2012; Fleming & Luo, 2013; Olson et al. 2016). It is thought that deformations in the membrane of these mechanoreceptor cells gate mechanosensitive ion channels, thus converting the mechanical energy into electrical activity. Together, these data suggest that the ability of mechanoreceptors to detect mechanical stimuli depends on the presence of the mechanosensible ion channels within them (see Del Valle et al. 2012). Consistent with this view, ion channel mechanosensor candidates have been found in mechanoreceptive neurons and mechanoreceptors (Del Valle et al. 2012; Delmas & Coste, 2013; Ranade et al. 2015), but studies in humans are scarce (Calavia et al. 2010; Cabo et al. 2015; Alonso-González et al. 2017).

In this study, we investigated the occurrence and distribution of Piezo2 in mechanosensory nerve formations of human digital skin, especially Merkel cell–neurite complexes and Meissner and Pacinian corpuscles. Merkel cell–neurite complexes are tactile organs consisting of Merkel cells and afferent SA-LTMR endings that mediate responses to fine touch (see Woo et al. 2015a). Meissner's corpuscles (Vega

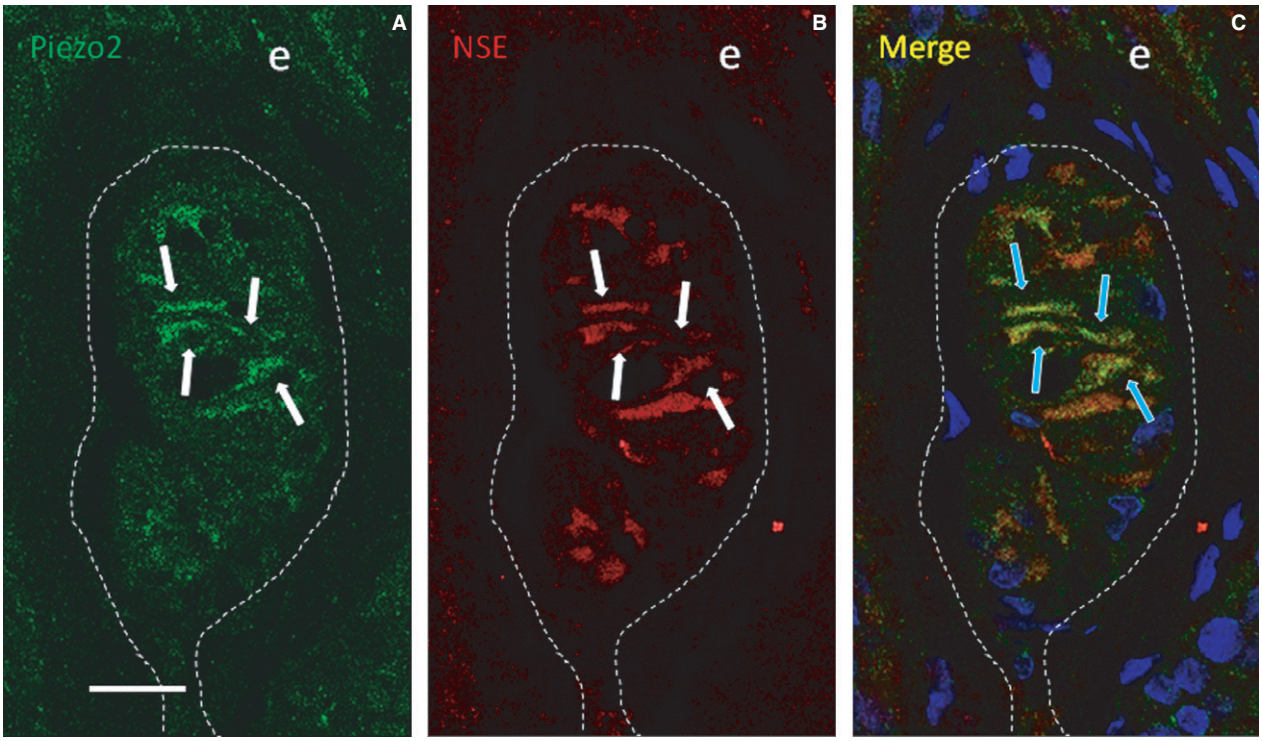

Fig. 6 The axon of human Meissner's corpuscles positive for Piezo2. Double immunofluorescence for Piezo2 using anti-Piezo2 polyclonal conjugated with Alexa fluor 488 (A, green fluorescence) and anti-NSE conjugated with Cy3 (B, red fluorescence) in human digital Meissner's corpuscles. Piezo2 were co-localized with axonic marker (NSE) only on axon segments (C, blue arrows). The white dotted line delineates the profile of Meissner's corpuscles. (A–C) Objective 63×/1.40 oil; pinhole AU 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm. Scale bar: 10 μm. e, epidermis.

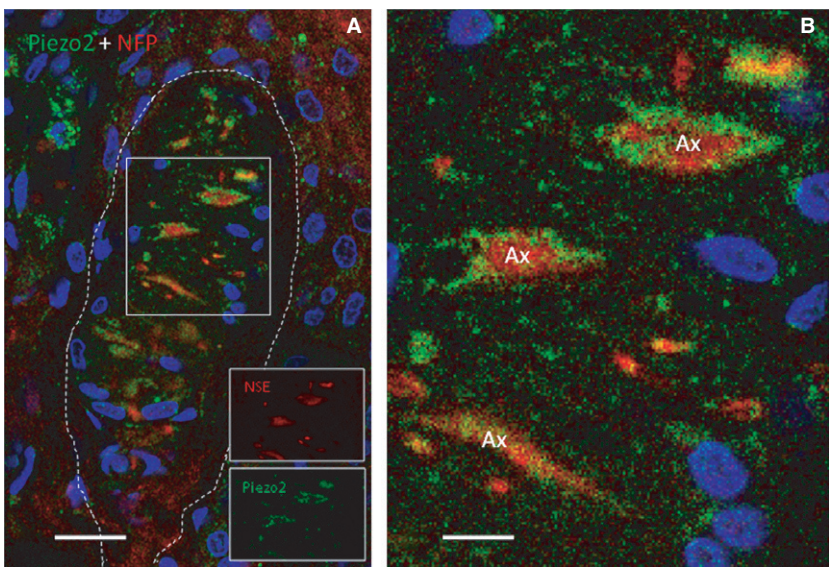

Fig. 7 Piezo2 is presumably situated in the axolemma of Meissner's corpuscles. Immunohistochemical localization of Piezo2 using anti-Piezo2 polyclonal conjugated with Alexa fluor 488 (square in a, green fluorescence) and anti-NF monoclonal conjugated with Cy3 (square in a, red fluorescence) in human digital Meissner's corpuscles. When the images overlapped, there was no co-localization of Piezo2-NFP (A). The square delimits the enlarged observation area (B). Piezo2 is localized at the periphery of the axonic marker (anti-NF), presumably in the axolemma. The white dotted line delineates the profile of Meissner's corpuscles. (A,B) Objective 63×/1.40 oil; pinhole AU 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm. Scale bar: 10 μm. Ax, axon.

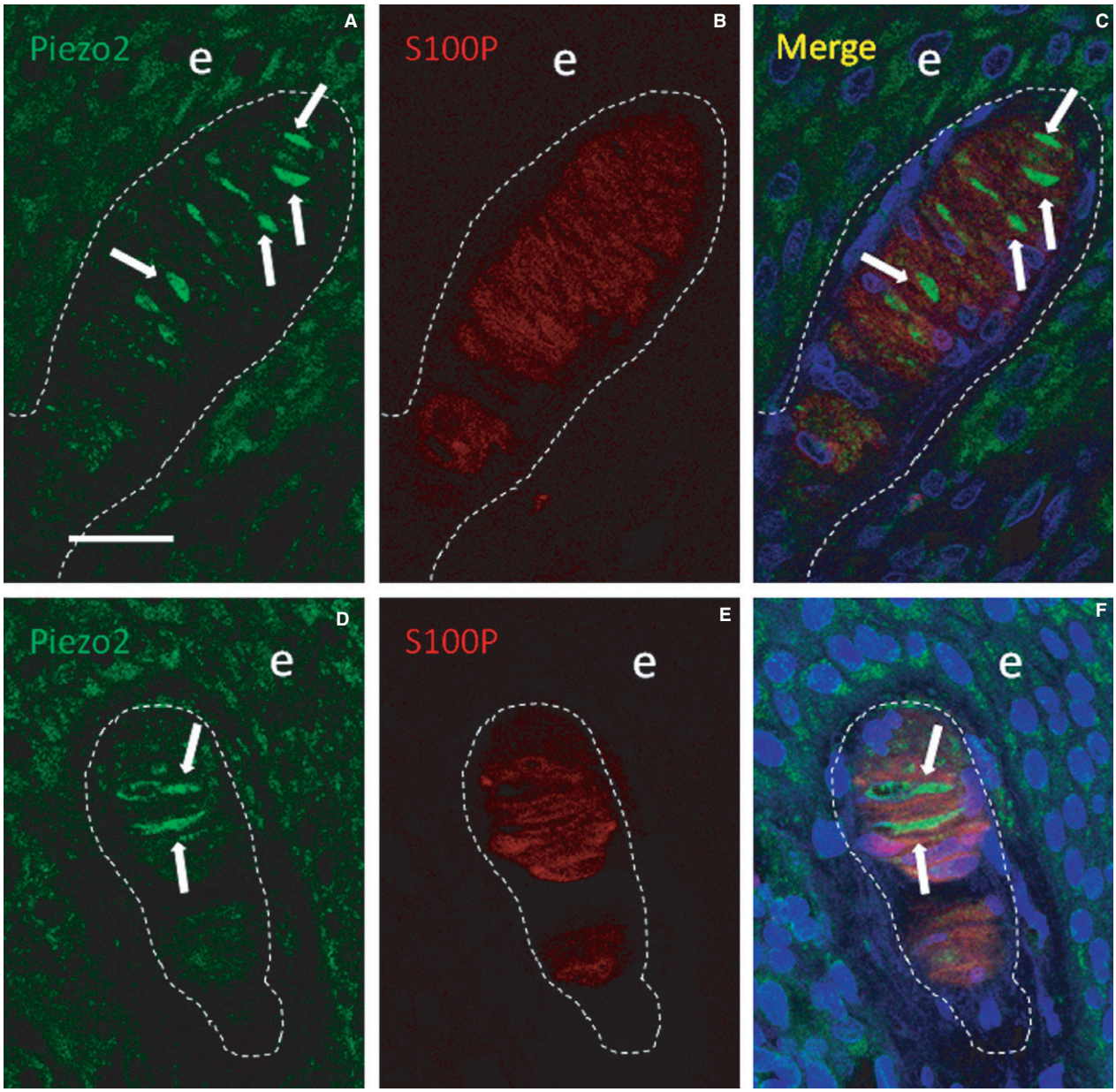

Fig. 8 Piezo2 is not present in lamellar cells of Meissner's corpuscles. Double immunofluorescence for Piezo2 (A,D, green fluorescence) and anti-S100 monoclonal (lamellar cells marker) (B,E, red fluorescence) in human digital Meissner's corpuscles. There was no co-localization of Piezo2 and S100 (C,F). The axon is indicated by the white arrow. The white dotted line delineates the profile of Meissner's corpuscles. (A–C) Objective 63×/1.40 oil; pinhole AU 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm. Scale bar: 10 μm. e, epidermis.

et al. 2012) are supplied by the peripheral branch of RA-LTMR sensory nerve fibres that are activated by innocuous mechanical forces applied to the skin, although some can also be activated by noxious stimuli (Johnson, 2001; Abraira & Ginty, 2013; Fleming & Luo, 2013). Finally, Pacinian corpuscles function as RA-LTMRs (Li et al. 2011) that primarily detect gross pressure changes and vibrations (Johnson, 2001; Roudaut et al. 2012; Fleming & Luo, 2013; Zimmerman et al. 2014). Data from cultures of dorsal root ganglion neurons from Piezo2-conditional knockout mice and from *ex vivo* skin nerve preparations show that the mechanosensitivity of LTMRs strongly depends on Piezo2 (Ranade et al. 2014). To our knowledge, the presence of Piezo2 has never been reported in human mechanosensory structures.

Human Merkel cells display strong immunoreactivity for Piezo2. To characterize them and their relationship with nerve fibres, we used a battery of antibodies against specific cytoskeletal, synaptic vesicles, axons and Schwann cells, as Merkel cells form synaptic-like contacts with sensory afferent terminals (Llombart et al. 2005; Maksimovic et al.

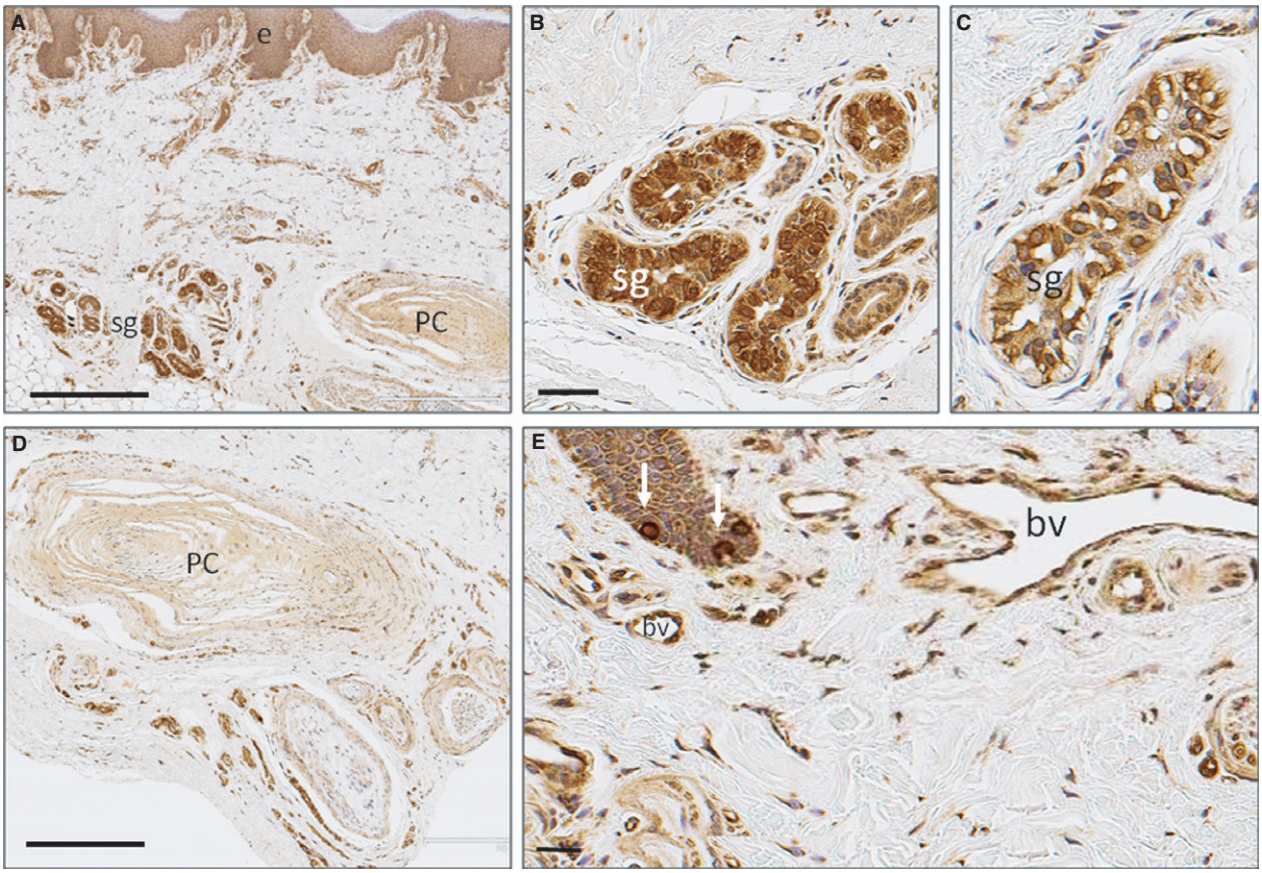

Fig. 9 Piezo2 in other cutaneous structures and sensory corpuscles. Single immunohistochemistry for Piezo2 was negative in Pacinian corpuscles (A,D) and positive in superficial and deep dermal nerves axons (A). In the dermis, especially the basal keratinocytes, there were occasionally immunoreactive cells showing a faint cytoplasmic pattern (A,E), and both the endothelial and muscular layers of blood vessels were Piezo2-positive (E), as were the acini and ductal cells of the sweat glands (A–C). The white arrow shows Piezo2-positive Merkel cells. Scale bar: 500 μm (A,D), 40 μm (B,C,E). Bv, blood vessels; e, epidermis; PC, Pacinian corpuscle; sg, sweat gland.

2013; Fukuhara et al. 2016). We demonstrated they are CK20, which is in line with the epithelial (Morrison et al. 2009; Van Keymeulen et al. 2009) and non-neural crest (Szeder et al. 2003) origin of these cells. Nevertheless, we have also found NSE immunostaining in some Merkel cells, as previously reported (Masuda et al. 1986; Isgrò et al. 2015), which argues for a neuronal instead of epithelial origin. Thus, the immunohistochemical approach to Merkel cells does not clarify their filiation. Interestingly, a subset of morphologically and immunohistochemically characterized Merkel cells were Piezo2-negative. At present, this finding cannot be explained, and it is unknown whether the Piezo2-negative Merkel cells act as mechanoreceptors. On the other hand, the expression of Piezo2 by Merkel cells was independent of its innervation, as nerve profiles were found to be associated with both Piezo2-positive and Piezo2-negative Merkel cells. It must be emphasized that Piezo2-positive nerve profiles were never found. Therefore, the functions of Piezo2 in the Merkel cell–neurite complexes seemed to be centred in the cells rather than in the nerve terminals. Our results are in good agreement with data demonstrating that Merkel cells are mechanosensitive cells (Maricich et al. 2009) and function in touch transduction via Piezo2 (Woo et al. 2014). However, controversy exists as to whether Merkel cells or sensory axons are the sites of mechanotransduction (Ikeda et al. 2014; Ranade et al. 2014; see Woo et al. 2015a), although our results lend support to the first opinion. Some studies have suggested that both components of the Merkel cell–neurite complexes participate in discriminative touch: Merkel cells signal static stimuli, such as pressure, whereas sensory afferents transduce dynamic stimuli, such as moving gratings (Maksimovic et al. 2014; Nakatani et al. 2015).

The axons supplying Meissner's corpuscles were also Piezo2-positive, whereas the Schwann cell-related lamellar cells forming these sensory structures were negative. These results match previous data in murine Meissner-like corpuscles (Ranade et al. 2014). Thus, in these sensory structures, Piezo2 is localized in the nerve terminal instead of the accessory cells. Other kinds of mechanoreceptors, such as

Ruffini or Pacinian corpuscles, were devoid of Piezo2 immunoreactivity, suggesting that this mechanoprotein is not involved in their physiology.

The two kinds of Piezo2-positive cutaneous mechanoreceptors identified in the present study comprise afferent terminals associated with non-neuronal cell types, such as Merkel cells and modified Schwann cells. An open question is whether these non-neuronal cells serve primarily as passive mechanical filters or actively participate in mechanosensory transduction. It seems evident that Piezo2 is present only in the non-neuronal cells of the Merkel cell–neurite complexes, whereas it is restricted to the axons and absent from the non-neuronal cells in Meissner's corpuscles. Nevertheless, these non-neuronal cells in Meissner's corpuscles (ASIC2 and TRPV4; Cabo et al. 2015; Alonso-González et al. 2017) and the Merkel cell–neurite complexes display immunoreactivity to other putative mechanoproteins that have been found in Merkel cells (ASIC2, Cabo et al. 2015; Piezo2 present results) and Schwann cells. Thus, the expression of mechanoproteins by human cutaneous mechanoreceptor cells remains to be clarified in future studies, especially as to why the same protein is present in some cases in non-neuronal cells and in other cases in the LTMR terminal.

Although functional studies are required in humans, the present data suggest that Piezo2 might be required for innocuous fine touch, whereas it does not participate in detecting rough touch or vibration, as it was absent from Pacinian corpuscles. This opinion is supported by data obtained in Piezo2-deficient animals, which show an almost complete deficit in light-touch sensation without changes in other somatosensory functions, demonstrating that Piezo2 is responsible for the mechanosensitivity SA- (Merkel cells) and RA-LTMRs are involved in innocuous touch sensation (Ranade et al. 2014).

Outside the mechanoreceptors, we observed Piezo2 immunoreactivity in non-nervous tissue such as epidermal cells, endothelial cells of blood vessels, and cells in sweat glands. Although the precise role of Piezo2 in those cells remains to be established, it has been demonstrated that Piezo proteins, principally Piezo1, participate in some mechano-associated biological processes, such as sensing of shear stress, regulation of urine flow and bladder distention, volume regulation, and cellular development, migration, proliferation and elongation (Bagriantsev et al. 2014; Wu et al. 2017). Further studies should be carried out, especially in subjects carrying mutations in Piezo2, to clarify the role of Piezo2 in these tissues.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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3.4. Resultados – Publicación 4

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Glans clitoris innervation: PIEZO2 and sexual mechanosensitivity

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Abstract

The clitoris is a leading player in female sexual arousal, if not the main protagonist. Despite this role, studies performed on this structure with specific neuroanatomical techniques are few. This study focuses on glans clitoris innervation, with special emphasis on sensory corpuscles and the presence of the mechanotransducer protein PIEZO2 in these structures. Six glans clitoris samples were obtained at autopsy covering an age spectrum between 52 and 83 years old. Several types of nerve terminations including free nerve endings, genital endbulbs as well as Meissner-like corpuscles and Pacinian corpuscles, but not Ruffini corpuscles, were found. Although corpuscular morphology in the glans clitoris was subtly different from the cutaneous digital counterparts, their basic composition was comparable for both Pacinian and Meissner-like corpuscles. Genital endbulbs showed heterogeneous morphology, and the axons usually exhibited a typical "wool ball" or "yarn ball" aspect. Some of them were lobulated and variably encapsulated by endoneurial elements (65%); from the capsule originate septa that divides the genital endbulbs, suggesting that they are found in clusters rather than as single corpuscles. In addition, most corpuscles in the glans clitoris showed axonal PIEZO2 immunoreactivity, thus, suggesting a mechanical role and molecular mechanisms of mechanosensibility similar to those of digital Meissner's corpuscles. Our results demonstrate that sensory corpuscles of the glans clitoris are similar to those of other glabrous skin zones, as most genital organs are characterized by clusters of corpuscles and the occurrence of the mechanoprotein PIEZO2 in the axons. These findings strongly suggest that PIEZO2 participates in erotic and sexual mechanical sensing.

KEYWORDS
clitoral innervation, clitoris, glabrous skin, PIEZO2, sensory corpuscles

1 | INTRODUCTION

Vertebrate skin contains specialized sensory organs collectively referred to as sensory corpuscles, which are supplied by peripheral processes of primary sensory neurons that encode non-painful mechanical stimuli (low-threshold mechanoreceptors, LTMRs; Abraira & Ginty, 2013; Fleming & Luo, 2013; Zimmerman *et al.*, 2014). A group of these structures localized in the erogenous zones are involved in sexual pleasure.

In women, the glans clitoris is generally considered the structure most involved in sexual pleasure, and it is a key element required to reach orgasm. It is a fibrovascular, non-erectile, densely innervated structure located in the midline that is the only

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external manifestation of the clitoris (O'Connell *et al.*, 2005; Pauls, 2015; Puppo & Puppo, 2015; Jackson *et al.*, 2019). It is covered by specialized glabrous skin, fine-tuned for sexual pleasure sensations and reproductive reflexes. This particular skin specialization is associated with different combinations of LTMRs, rendering it neurophysiologically and functionally distinct from the clitoris as a whole as the centre for triggering the orgasmic response (Pauls, 2015). However, it has other reproductive functions. In fact, stimulation of the clitoris activates the brain to instigate changes in the female genital tract which are of major importance in facilitating reproductive success (Levin, 2020).

The macroscopic innervation of the glans clitoris occurs primarily via the dorsal nerve of the clitoris, a branch of the pudendal nerve, with contributions from the cutaneous branch of the ilioinguinal nerve, the genital branch of the genitofemoral nerve and the perineal branch of the posterior femoral cutaneous nerve (Pauls, 2015; Yeung & Pauls, 2016). Microscopic innervation of the glans clitoris was analysed by classical neuroanatomists for more than 100 years using silver impregnation methods, and different morphotypes of sensory corpuscles were described. All these studies were compiled by Seto (1963) in his classical book "Studies on the sensory innervation (human sensibility)." He affirms that the adult human clitoris contains abundant "genital nerve bodies" divided into three types, as well as Pacinian corpuscles, adjacent to branched nerve endings. More recently, immunohistochemical techniques identifying specific markers for axonal and Schwann-related cells were used (Vega *et al.*, 2009). Shih *et al.* (2013), using immunohistochemistry for the axon and glial cells, identified some Pacinian corpuscles and abundance of corpuscular receptors (genital endbulbs) within the glans clitoris, variably arranged in the subepithelial tissues. In the monograph "Anatomic study of the clitoris and the bulbo-clitoral organ," Di Marino and Lepidi (2014) report that the human glans clitoris contains standard tactile corpuscles including Meissner's, Ruffini's and Pacinian corpuscles, as well as corpuscles specialized to the clitoris such as Krause corpuscles. Meissner-like corpuscles and genital endbulbs have been also described in the labia minora (Feito *et al.*, 2018).

Since the glans clitoris is covered by thin glabrous skin, it may be assumed that the sensory nerve endings share the structure and immunohistochemical properties of digital ones. The periaxonic cells that form sensory corpuscles are continuous with the cells of nerve trunks, except for the perineurium which can be either present (Pacinian corpuscles) or absent (Meissner's corpuscles; Vega *et al.*, 2009; Feito *et al.*, 2016; García-Piqueras *et al.*, 2017, 2020). Thus, the first goal of this study was to analyse whether the sensory corpuscles present in the glans clitoris share basic immunohistochemical characteristics with the digital ones, including a capsule of endoneurial and/or perineurial filiation.

In sensory corpuscles, conversion of mechanical stimuli into electrical signals involves mechanogated ion channels. Recent studies have shown that PIEZO2 is required for mechanotransduction in mammalian cells (Coste *et al.*, 2010) and is expressed in LTMRs of mammalian dorsal root ganglia (Ranade *et al.*, 2014; Honoré *et al.*, 2015). PIEZO2 is also present in Merkel discs and isolated Merkel cells, as well as in Meissner's corpuscles (Ikeda *et al.*, 2014; Maksimovic *et al.*, 2014; Ranade *et al.*, 2014; Woo *et al.*, 2014; García-Mesa *et al.*, 2017; García-Piqueras *et al.*, 2019). As far as we know, PIEZO2 expression in genital sensory corpuscles has not been reported, despite the fact that genital tactile stimulation is a critical component of sexual arousal. Furthermore, there is general agreement that it is possible to have an orgasm through direct simulation of the glans clitoris (Jannini *et al.*, 2012). Therefore, the second aim of this study was to analyse whether PIEZO2 is the mechanotransducer present in these sensory structures.

2 | METHODS

2.1 | Tissue processing

Six glans clitoris from un-embalmed female cadavers were obtained from our laboratory collection (Registro Nacional de Biobancos, Sección colecciones, Ref. C-0001627), and the study was approved by the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. CElm, PAst: Proyecto 266/18). All these materials were obtained in compliance with Spanish law (RD 1301/2006; Ley 14/2007; DR 1716/2011; Orden ECC 1414/2013). The age range was 52–83 years. These materials were routinely embedded in paraffin and cut into serial sections 10 μm thick.

2.2 | Single immunohistochemistry

Deparaffinized and rehydrated sections were processed for indirect immunohistochemistry using Leica Bond™ Polymer Refine Detection Kit (Leica Biosystems™, Newcastle, UK) following the manufacturer's instructions. Because of the continuity between the axon and periaxonic cells of the sensory corpuscles and the cells of the nerve trunks, we used immunohistochemistry to examine the expression of axonal (neuron-specific enolase: NSE), Schwann-related cell (S100 protein: S100P), endoneurial (CD34 antigen) or perineurial (Glucose transporter 1: Glut1) markers in the glans clitoris. Furthermore, we used an antibody against PIEZO2 to detect this mechanoprotein. Table 1 summarizes the primary antibodies used in the study to examine the various corpuscular constituents. Indirect immunohistochemistry included several negative and positive controls as well as internal positive and negative controls.

2.3 | Double immunofluorescence

In deparaffinized and rehydrated sections, non-specific binding was reduced (incubation for 30 minutes with a solution of 5% bovine serum albumin in Tris-buffered saline (TBS), pH 7.4). Sections were then incubated overnight at 4°C in a humid chamber with a 1:1 (v/v)

TABLE 1 Primary antibodies used in this study

Antigen	Origin	Dilution	Supplier
CD34 (clone QB-END/10)	Mouse	Prediluted	Master Diagnostica[a]
Glut1	Rabbit	0.5 μg/ml	Cell Marque[b]
NSE (clone BBS/NC/VI-H14)	Mouse	1:1000	Dako[c]
NFP (clone 2F11)	Mouse	1:100	Dako[c]
PIEZO2[f]	Rabbit	1:200	Sigma-Aldrich[d]
S100 protein (clone 4C4.9)	Mouse	1:1000	ThermoFisher Scientific[e]
S100 protein	Rabbit	1:1000	Dako[c]

Abbreviations: Glut1, glucose transporter 1; NFP, neurofilament protein; NSE, neuron-specific enolase.

[a]Granada, Spain

[b]Seattle, WA, USA

[c]Glostrup, Denmark

[d]Saint Louis, MS, USA

[e]Freemont, CA, USA

[f]Amino acid sequence recognized: FEDENKAAVRIMAGDNVEICMNLDAASFSQHNP.

mixture of anti-S100P and anti-NSE; anti-S100P and anti-CD34; anti-S100P and anti-Glut1; anti-S100P and anti-PIEZO2; or anti-NSE and anti-PIEZO2. The dilutions of the antibodies for double immunofluorescence were as in Table 1. After rinsing, sections were incubated for 1 hour with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Serotec™, Oxford, UK, diluted 1:1000), rinsed again and incubated for another hour with a Cy3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch™, Baltimore, MD, USA, diluted 1:50). Both steps were performed at 20°C room temperature in a dark, humid chamber. Thereafter, sections were washed and mounted with Fluoromount Gold (ThermoFisher, Runcoen, UK), and finally, sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole; 10 ng/ml) to label nuclei. Triple staining was detected using a Leica DMR-XA automatic fluorescence microscope coupled with Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany), and captured images were processed using the software ImageJ, version 1.43 g at the Master Biophotonics Facility, McMaster University, Ontario, Canada (www.macbiophotonics.ca). As controls, representative sections were processed in the same way as described above, using non-immune rabbit or mouse sera instead of primary antibodies or while omitting primary antibodies during incubation.

2.4 | Quantitative analyses

Ten sections of glans clitoris samples, 10 μm thick, 100 μm apart, were processed for S100P + CD34 immunohistochemistry and used to identify sensory corpuscles. The sections were scanned by an SCN400F scanner (Leica Biosystems), and the scans were

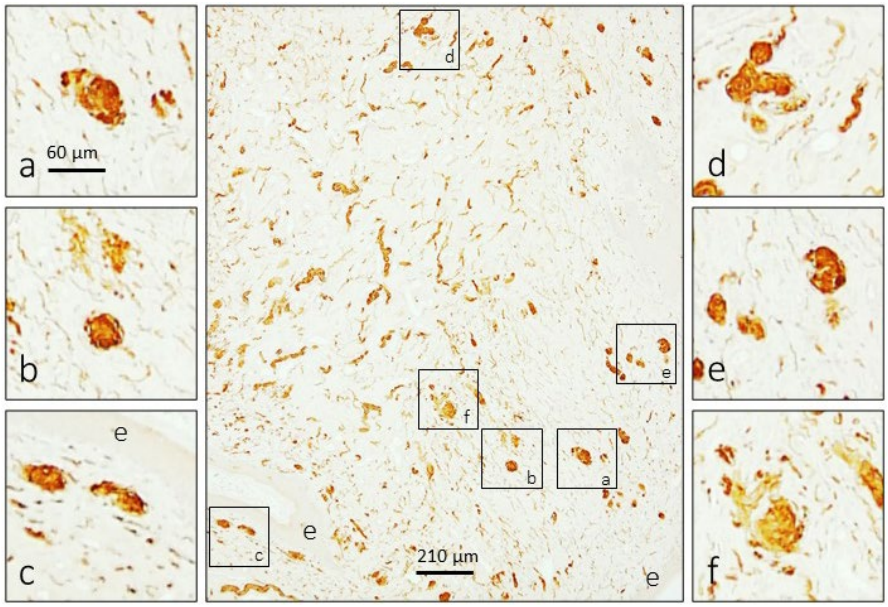

FIGURE 1 Immunohistochemical detection of S100 protein in the human glans clitoris. The glans clitoris contains a dense network of nerve fibres, the ends of which can form different morphotypes of sensory corpuscles localized subepithelially but also in the central part of the organ. e: epithelium

computerized using SlidePath Gateway LAN software (Leica Biosystems™). Then, in five randomly selected fields of 400 μm^2 each per section (Figure S1a,b), the number of sensory corpuscles were counted by two independent observers (YG-M and JAV). Values are expressed as the mean of sensory corpuscles by mm^2. Due to the low number of sampled corpuscles, no statistical analysis was carried out.

3 | RESULTS

The glans clitoris is richly innervated by nerve fibres of different diameters that form a network apparently denser at the periphery than in the central part of the organ. These fibres form perivascular plexuses, terminate as free nerve endings (especially in dermal papillae) and form different morphotypes of sensory corpuscles (Figure 1).

3.1 | Morphotypes of sensory corpuscles

Sensory corpuscles in the glans clitoris were abundant and of variable morphology and size. They consisted of a very tightly coiled axon (with a "wool ball" or "yarn ball" appearance), with remarkable differences in the diameter of axonal branches, embedded in densely packed Schwann-like cells.

Sensory corpuscles localized immediately beneath the epithelium were smaller than deeper ones and showed an irregular and fragmented aspect (Figure 2a–c). Despite their localization, axon morphology (Figure 3a,b) and arrangement of Schwann-related cells differed from those of typical cutaneous Meissner corpuscles. Sensory corpuscles localized in deeper subepithelial tissues (Figures 2d–i and 3c–f), as well as those located in the inner part of the glans clitoris, showed the typical morphology of the so-called genital endbulbs. The size of these bodies was very variable, but there was a tendency to increase in size from surface to deeper regions of the skin (see Figure 2).

The intricate relationship between axons and Schwann-related cells, independently of corpuscular location and size and leading to a complex arrangement of both structures, is depicted in Figures 4 and 5.

One characteristic aspect of genital endbulbs was the apparent lobulation observed in about 60% of them. We performed immunohistochemistry for CD34 (an endoneurial marker) and Glut1 (a perineurial marker) to investigate whether this lobulation is real or not. Approximately 65% of the lobulated genital endbulbs presented endoneurial CD34-positive septa dividing the corpuscles (Figure 6a–c); the remaining (approximately 35%) lacked an endoneurial capsule, and CD34 positivity was restricted to the endothelial cells of the capillaries that encircled the corpuscle (Figure 6d–f). In the dermis immediately below the epidermis, both capsulated and non-capsulated genital endbulbs were observed (Figure 6g–i). Immunoreactivity for Glut1 was generally absent covering genital endbulbs, although it was positive in the perineurium of nerve trunks and the capsule of Pacinian corpuscles (data not shown).

In addition to Meissner-like corpuscles and genital endbulbs, well-differentiated Pacinian corpuscles were observed in the central part of the glans clitoris (Figure 7). They showed an irregular morphology, but all their components were present (axon, inner-core Schwann-related cells, CD34-positive intermediate layer and well-developed capsule) with their typical immunohistochemical profile (Figure 7), including Glut1 immunoreactivity in the capsule.

3.2 | Density of sensory corpuscles

The estimated density of sensory corpuscles in sections immunostained for S100P was 4.37 ± 0.7 mm^2, and there was a trend in

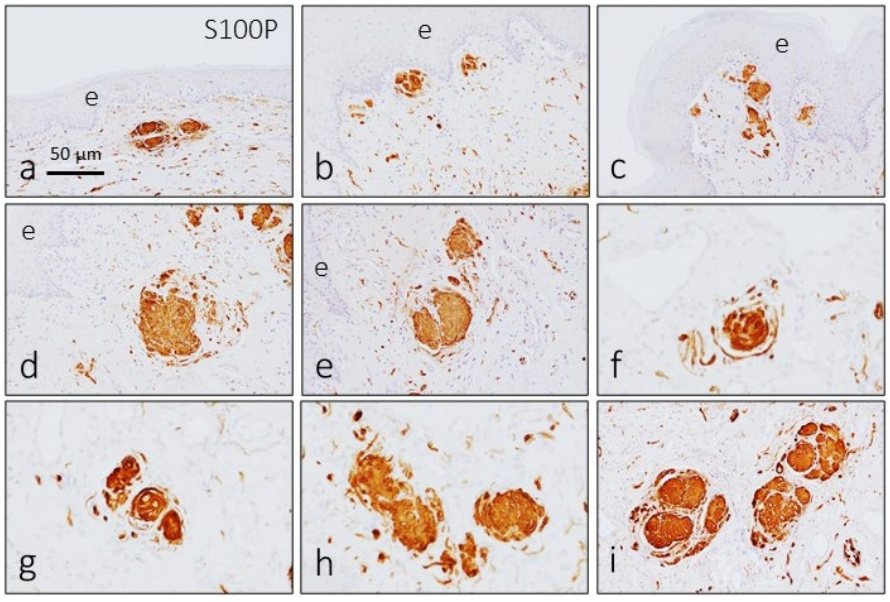

FIGURE 2 Immunohistochemical detection of S100 protein in the sensory corpuscles of the human glans clitoris. The Schwann-related cells of glans clitoris sensory corpuscles display a strong immunoreactivity for S100 protein. Sensory corpuscle morphology was variable, and the size increased from the subepithelial zone (a–c) to the intermediate zone (d, e) and deep zone (f–i). Some of the sensory corpuscles were lobulated. e, epithelium

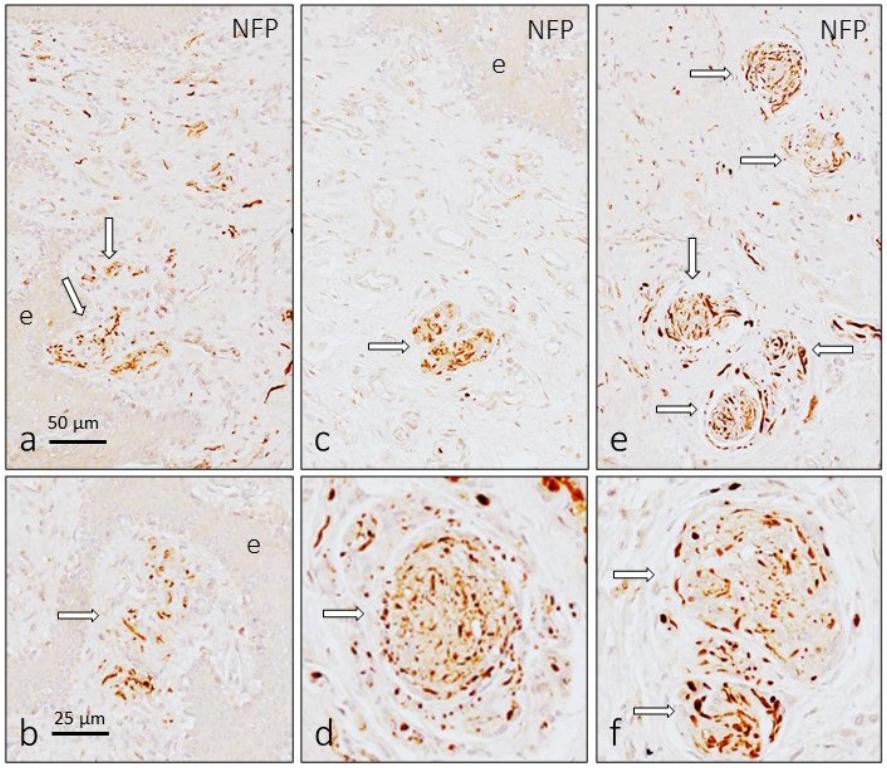

FIGURE 3 Immunohistochemical detection of neurofilament protein and neuron-specific enolase in the sensory corpuscles of the human glans clitoris. The arrangement of the axons in the dermal papillae was similar to that of cutaneous Meissner corpuscles (a and b), whereas in those placed in deep subepithelial tissues and in the central part of the organ (c–f), the axon was very tightly coiled and look like a "wool ball" or "yarn ball" with remarkable differences in the diameter of the axonal profiles

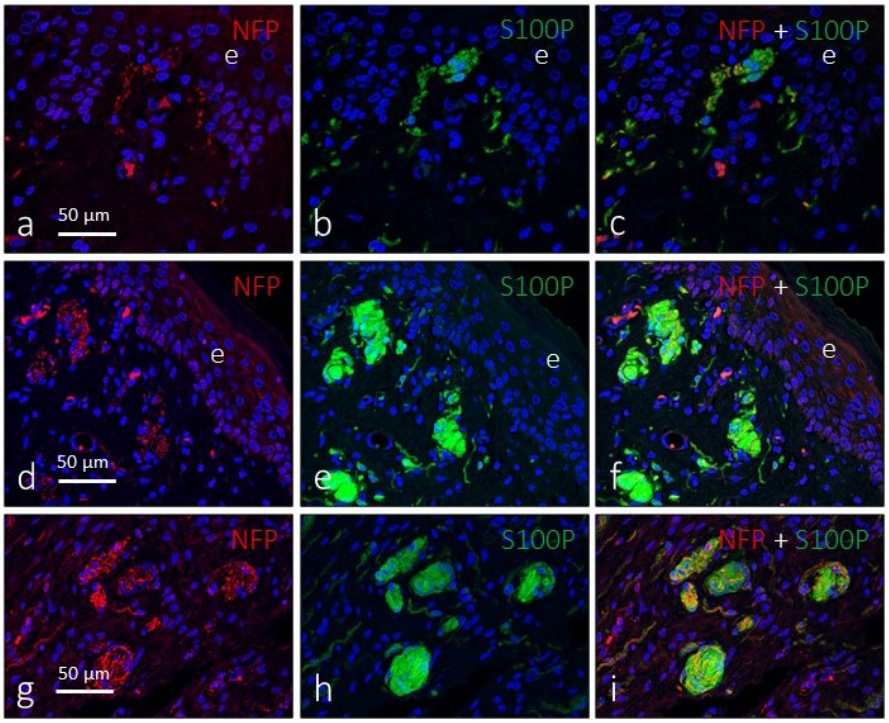

FIGURE 4 Double immunofluorescence for S100 protein (green fluorescence) and neurofilament protein (red fluorescence) in subepithelial (a–f) and intermediate (g–i) sensory corpuscles in the glans clitoris. Sections were counterstained with DAPI to ascertain structural details. Objective 63×/1.40 oil; pinhole 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm. e, epithelium

the density of sensory corpuscles that suggested an age-dependent decline. In fact, in sections from individuals aged 52–60 years, the mean values were 5.22 ± 0.4 mm^2, whereas in the sections from subjects aged 61–83 the mean values were 4.16 ± 0.8 mm^2 (Figure S1a,b). Nevertheless, these values must be taken with caution since numbers were obtained from sections immunostained for S100P. Furthermore, when a lobulated corpuscular structure is seen in sections processed for simultaneous detection of S100P and CD34, if each lobule is considered to be an independent corpuscle, the mean values reach 13.9 ± 1.3 mm^2 (Figure S1c–e).

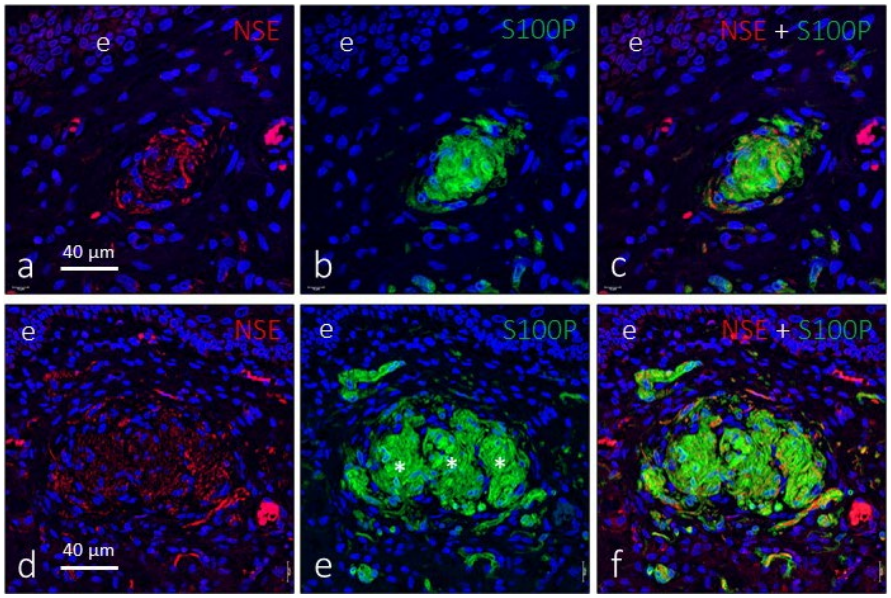

FIGURE 5 Double immunofluorescence for S100 protein (green fluorescence) and neuron-specific enolase (red fluorescence) in genital endbulbs of the deep zone of the glans clitoris. Some corpuscles seem to be lobulated (asterisks in e), and typically the axons are arranged in a "wool ball" or "yarn ball" shape with remarkable differences in the diameter of the axonal profiles. Sections were counterstained with DAPI to ascertain structural details. Objective 63×/1.40 oil; pinhole 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm

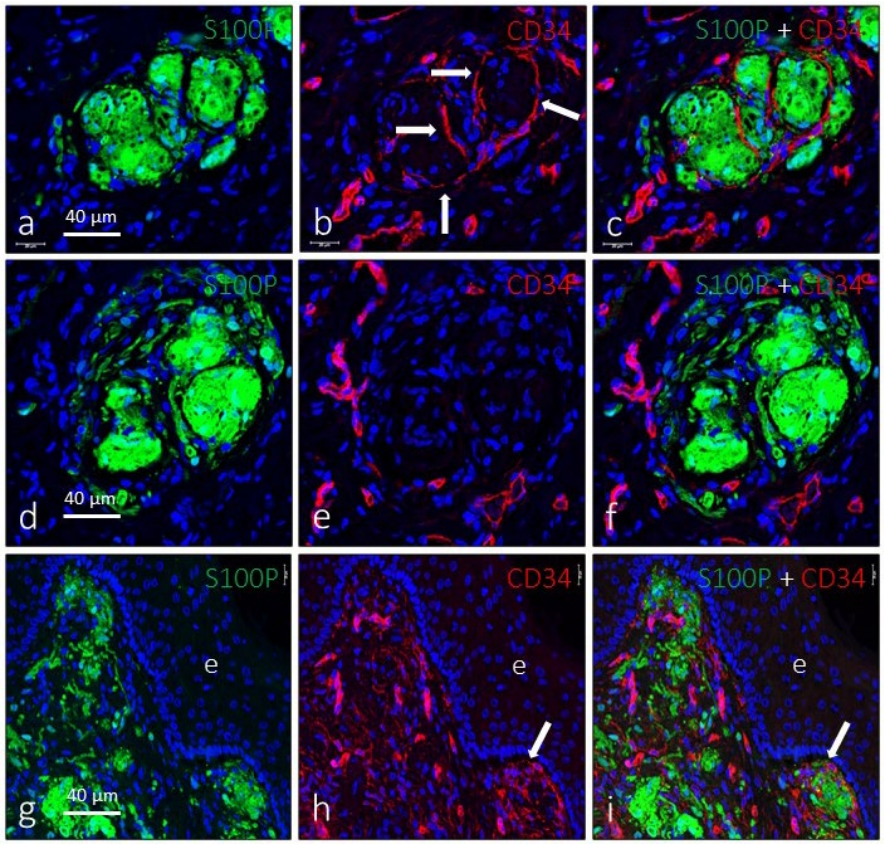

FIGURE 6 Evidence of lobulation of the genital endbulbs. Dual immunofluorescence for S100 protein (green fluorescence) and CD34 (red fluorescence) in deep (a–f) and subepithelial (g–i) sensory corpuscles. In some corpuscles (a–c), a well-defined lobulation indicated by the presence of CD34-positive septa was observed (arrows in b), whereas in others no evidence of capsulation or internal lobulation was observed (d–f). Subepithelial corpuscles were seen with either capsulation or no capsulation (g–i). Sections were counterstained with DAPI to ascertain structural details. Objective 63×/1.40 oil; pinhole 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm

3.3 | Sensory corpuscles of the glans clitoris contain PIEZO2

It is generally accepted that activation of mechanically gated ion channels, especially PIEZO2, is at the origin of the detection of low- or high-threshold mechanical stimuli (Ranade *et al.*, 2014). The presence of PIEZO2 in the glans clitoris was investigated using simple and double immunohistochemistry. PIEZO2 was detected in scattered cells localized in cells of the basal layer of the superficial epithelium, which were identified as Merkel cells based on their localization and morphology (Figure 8a,b). Furthermore, PIEZO2 immunoreactivity was observed in all subepithelial sensory corpuscles, but especially in genital endbulbs (Figure 8). The pattern of distribution of PIEZO2 within these structures resembles "wool balls" or "yarn balls" (Figure 8c–e) and, as mentioned previously for conventional neuronal markers, the axonal profiles show different

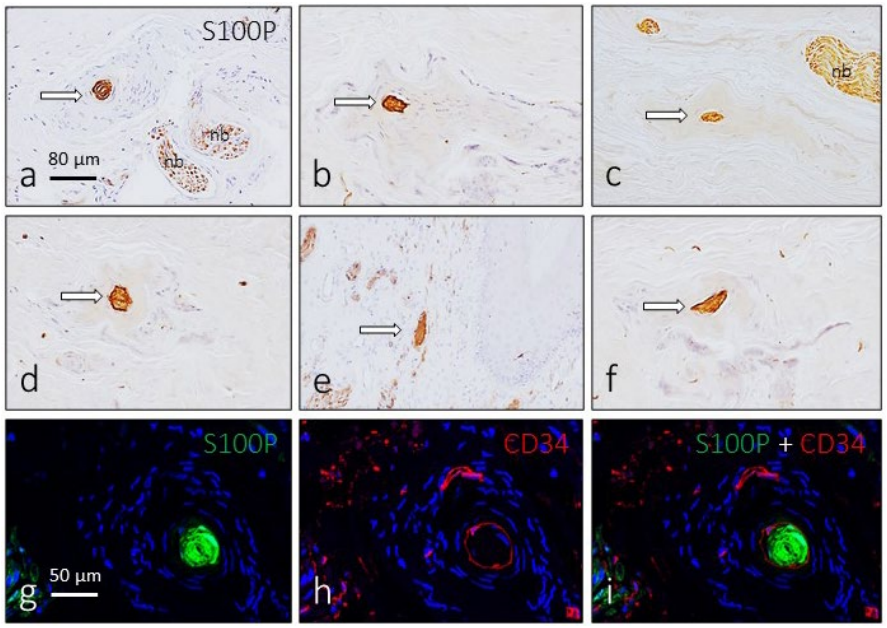

FIGURE 7 Pacinian corpuscles in the glans clitoris. In the central part of the organ, Pacinian corpuscles were regularly found. They showed an irregular morphology, but the axon, inner-core cells and capsule had a typical arrangement. Arrows in a–f indicate the inner core which was intensely immunoreactive for S100 protein. These corpuscles also displayed CD34 immunofluorescence in the intermediate layer (g–i). Sections g–i were counterstained with DAPI to ascertain structural details. Objective 63×/1.40 oil; pinhole 1.37; XY resolution 139.4 nm and Z resolution 235.8 nm. nb: nerve bundle

calibres. To confirm the axonal localization of PIEZO2, we performed double immunofluorescence with Schwann-like and axonal markers. PIEZO2 never co-localized with S100P (Figure 8f–h) and partially co-localized with neuron-specific enolase, especially in the larger axonal profiles (Figure 8i–k).

4 | DISCUSSION

The skin is a multisensory organ that receives profuse sensory innervation from primary sensory neurons placed in the peripheral sensory ganglia (McGlone & Reilly, 2010; McGlone *et al.*, 2014; Owens & Lumpkin, 2014). Cutaneous afferents can be distinguished anatomically based on their sensory terminals, or sensory corpuscles, and can be functionally classified based on the conduction speed of their action potentials (Rice & Albrecht, 2008; Gardner & Johnson, 2013).

The glans clitoris is covered by glabrous skin, which according to our results contains three main morphotypes of sensory corpuscles: Meissner-like corpuscles, genital endbulbs and Pacinian corpuscles. These data are in good agreement with previous reports (Seto, 1963; Shih *et al.*, 2013; Di Marino & Lepidi, 2014), especially with those form Shih *et al.* (2013). Conversely, we did not find Ruffini's or Krause corpuscles as reported by Di Marino and Lepidi (2014). Presumably, Meissner's and Pacinian corpuscles of the glans clitoris could be compared to their cutaneous counterparts and meet similar functions.

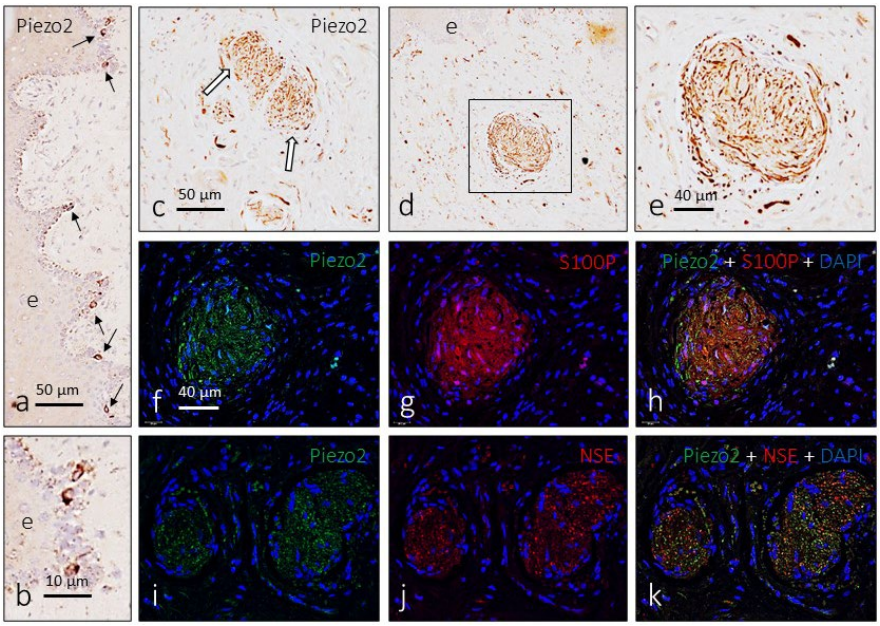

FIGURE 8 Immunodetection of PIEZO2 in the glans clitoris. PIEZO2 immunoreactivity is detected in cells of the epithelium basal layer (a and b) that were identified as Merkel cells (arrows in a). In the genital endbulbs, PIEZO2 shows a typical "wool ball" or "yarn ball" axonal pattern of distribution (c–e; arrows in c). Immunofluorescence confirmed that PIEZO2 is localized in the axon (i–k) and is absent from the Schwann-related cells (f–h)

Pauls (2015) affirms that the specialized glabrous skin of the glans clitoris contains combinations of LTMRs that make it functionally distinct, which ultimately determine the pleasure sensibility of the orgasm. In the words of Zimmerman *et al.* (2014) "Like individual instruments in an orchestra, each LTMR subtype conveys a specific feature of the forces acting on the skin, collectively culminating in a musical symphony of neural impulses that the brain translates as a touch." However, the sexual response to sexually arousing stimuli is not only mechanical but also motivates an incentive-based cycle comprising subjective experience and physiologic change (Basson, 2015).

Interestingly, although additional cases should be analysed to confirm our findings, we have found a trend in the density of sensory corpuscles that suggest a reduction with ageing, as occurs in the digital glabrous skin (García-Piqueras *et al.*, 2019). Nevertheless, we cannot affirm if all morphotypes are affected equally. These preliminary data seem oppose with studies that suggest that the clitoral sexual response and the female orgasm are not affected by ageing (Puppo, 2013); difficulty with orgasm in older women is often associated with a partner's erectile dysfunction (Granville and Pregler, 2018) rather than with age-related decline in orgasm. Additional studies enabling more precise clinical-histological correlation would likely be required to resolve this inconsistency.

The pattern of distribution of investigated antigens in the glans clitoris sensory corpuscles was identical to that in cutaneous ones. Axons were intensely immunoreactive for both NSE and neurofilament, and Schwann-related cells displayed strong S100P immunoreactivity. The arrangement of the axon in a "wool ball" or "yarn ball" pattern is consistent with the pictures reported in classical studies using silver impregnation or immunohistochemical techniques (Seto, 1963; Shih *et al.*, 2013; Di Marino and Lepidi, 2014). However, we added a new data regarding the structure of clitoral corpuscles: the occurrence of a capsule on endoneurial origin encircling the axon and the glial cells in 65% of genital endbulbs. Recently, we demonstrated that cutaneous Pacinian corpuscles (García-Piqueras *et al.*, 2017) as well as most Meissner's corpuscles (García-Piqueras *et al.*, 2020) have a CD34-positive intermediate layer and a capsule of endoneurial origin; corpuscular perineurial Glut1-positive derivatives are only present in Pacinian corpuscles (Feito *et al.*, 2016). Endoneurial capsulation in glans clitoris sensory corpuscles was very heterogeneous: there were both capsulated and non-capsulated structures, and, interestingly, in up to 65% of lobulated genital endbulbs there were internal septa dividing clusters of Schwann-related cells and balls of axons. Thus, this raised the question of whether the lobulated genital endbulbs are single sensory corpuscles or clusters of sensory corpuscles. Although one can assume that a single-axon branches and forms different "wool balls" based on these results alone, this concept should be revisited using 3D reconstructions from laser confocal records.

In LTMRs and the associated sensory corpuscles, mechanotransduction (i.e. conversion of mechanical stimuli into electrical signals) involves some potential mechanogated ion channels, which have been detected in both axon and Schwann-related cells (Calavia *et al.*, 2010; Cabo *et al.*, 2015; Alonso-González *et al.*, 2017), and PIEZO2 has emerged as the most likely candidate responsible for sensory mechanotransduction (Ranade *et al.*, 2014; García-Mesa *et al.*, 2017; García-Piqueras *et al.*, 2019). We have observed that the axon of glans clitoris sensory corpuscles displays PIEZO2 immunoreactivity similar to Meissner's corpuscles in human digital skin (García-Mesa *et al.*, 2017; García-Piqueras *et al.*, 2019). To our knowledge, the presence of mechanoproteins, and in particular PIEZO2, in the sensory corpuscles of genital organs is reported here for the first time. These findings are of interest since mechanical stimulation of the clitoris is essential for sexual arousal and orgasmic response (Jannini *et al.*, 2012; Pauls, 2015; Levin, 2020), and PIEZO2 is critical for mechanotransduction (Wu *et al.*, 2017). Nevertheless, it is important to keep in mind that women may experience other kind of orgasms in addition to the clitoral external orgasm (Jannini *et al.*, 2012). Beside sexual pleasure, clitoral mechanical stimulation may also represent a treatment method for female sexual dysfunction (Billups, 2002) or female stress urinary incontinence (Sønksen *et al.*, 2007).

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CONFLICTS OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

YG-M, LC and JM-C performed the experiments. JF and OG-S collected the material in compliance with ethical guidelines and performed part of the experiments. RC, and JG-P quantified the data. JF and JAV designed the study, analysed the data and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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3.5. Resultados – Publicación 5

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ORIGINAL PAPER

Sensory innervation of the human male prepuce: Meissner's corpuscles predominate

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Abstract

Meissner's corpuscles are the most abundant sensory corpuscles in the glabrous skin of the male prepuce. They are type I, rapidly adapting, low-threshold mechanoreceptors, and their function is linked to the expression of the mechanoprotein piezo-type mechanosensitive ion channel component 2 (PIEZO2). Stimulation of genital Meissner's corpuscles gives rise to sexual sensations. It has been recently demonstrated that digital Meissner's corpuscles, Meissner-like corpuscles, and genital end bulbs have an endoneurium-like capsule surrounding their neuronal elements; that is, the axon and glial lamellar cells, and their axons, display PIEZO2 immunoreactivity. It is unknown whether this is also the case for preputial Meissner's corpuscles. Furthermore, the expression of certain proteins that have been found in Meissner's corpuscles at other anatomical locations, especially in the digits, has not been investigated in preputial Meissner's corpuscles. Here, we used immunohistochemistry to investigate the expression of axonal (neurofilament, neuron-specific enolase), glial (S100 protein, glial fibrillary acidic protein, vimentin), endoneurial (CD34), and perineurial (glucose transporter 1) markers in the preputial and digital Meissner's corpuscles of male participants aged between 5 and 23 years. Furthermore, we investigated the occurrence of the mechanoprotein PIEZO2 in male preputial Meissner's corpuscles. Human male prepuce contains numerous Meissner's corpuscles, which may be grouped or isolated and are regularly distributed in the dermal papillae. Lamellar glial cells display strong expression of S100 protein and vimentin but lack expression of glial fibrillary acidic protein. In addition, they show axonal PIEZO2 expression and have an endoneurial capsule, but no perineurial. Our results indicate that human male preputial Meissner's corpuscles share the immunohistochemical profile of digital Meissner's corpuscles, which is considered to be necessary for mechanotransduction. These data strongly suggest that the structure and function of Meissner's corpuscles are independent of their anatomical location.

KEYWORDS
capsule, human, male prepuce, Meissner's corpuscles, PIEZO2

1 | INTRODUCTION

The male prepuce, also known as the foreskin, is a cutaneous structure that covers the glans penis to protect the external genitalia. It is involved in sexual sensations and arousal (Cold & Taylor, 1999). However, a study on the association of penile histology with sexual response found no basis for ascribing sexual function to the prepuce (Cox et al., 2015). The prepuce contains sensory corpuscles related to various modalities of mechanosensitivity. In 1956, Winkelmann summarized all existing knowledge on prepuce innervation and published an excellent study on the cutaneous innervation of the human neonatal prepuce. He described intraepithelial "fibrils," "papillary endings," and Vater-Pacini corpuscles, but not the classical dermal "bodies" such as Meissner's corpuscles, Krause end bulbs, Dogiel's bodies, Golgi-Mazzoni bodies, and genital corpuscles. Later studies confirmed the presence of free nerve endings, Pacinian corpuscles, and Merkel cells, although the most common preputial sensory corpuscles were Meissner's corpuscles (Bath et al., 2008; Cold & Taylor, 1999; Guo et al., 2007; Jiang et al., 2006; Malkoc et al., 2012; Martín-Alguacil et al., 2015; Taylor et al., 1996).

Certain preputial sensory corpuscles, such as Meissner's corpuscles, Pacinian corpuscles, and Merkel cell-neurite complexes, function as mechanoreceptors in human glabrous skin (Cobo et al., 2020, 2021; Zimmerman et al., 2014), mediating various functions related to mechanosensitivity, especially touch (McGlone & Reilly, 2010; McGlone et al., 2014). They have a functional relationship with peripheral processes of primary sensory neurons that encode non-painful mechanical stimuli (low-threshold mechanoreceptors [LTMRs]; Abraira & Ginty, 2013; Fleming & Luo, 2013; Zimmerman et al., 2014). Meissner's and Pacinian corpuscles correspond to type I and type II rapidly adapting LTMRs, whereas Merkel cell-neurite complexes and Ruffini corpuscles correspond to type I and type II slowly adapting LTMRs, respectively (Fleming & Luo, 2013; Rice & Albrecht, 2008; Zimmerman et al., 2014). Slowly adapting LTMRs are involved in the sensation of fine touch, and rapidly adapting LTMRs are involved in the sensations of vibration and motion across the skin (Johnson, 2001; Jones & Smith, 2014; Olson et al., 2016; Owens & Lumpkin, 2014).

The conversion of mechanical stimuli into electrical signals in sensory corpuscles involves mechano-gated ion channel activation. Recent studies have shown that piezo-type mechanosensitive ion channel component 2 (PIEZO2), a vertebrate stretch-gated multipass transmembrane protein that is a component of a certain mechanically gated ion channel, is required for mechanotransduction in mammalian cells (Coste et al., 2010; Honoré et al., 2015; Ranade et al., 2014). It is expressed in the dorsal root ganglia (Coste et al., 2010; Ranade et al., 2014), Merkel discs, isolated Merkel cells, and Meissner's corpuscles in murine and human skin (García-Mesa et al., 2017; García-Piqueras et al., 2019; Ikeda et al., 2014; Maksimovic et al., 2014; Ranade et al., 2014; Woo et al., 2014). To the best of our knowledge, the expression of PIEZO2 in preputial mechanoreceptors has not been investigated. However, Chen et al., (2020) studied the role of PIEZO2 in the mechanism underlying premature ejaculation in rats and reported that PIEZO2 expression was significantly increased in the penile head and dorsal root ganglia of these rats.

This study aimed to meticulously investigate the types of sensory corpuscles present in the human male prepuce and determine whether they expressed PIEZO2 mechanoproteins. The main aim of this study was to elucidate the molecular mechanisms underlying mechanosensitivity in sexual organs.

2 | METHODS

2.1 | Tissues

Prepuce samples were obtained from 32 boys and men (aged 5–23 years) who underwent routine circumcision due to phimosis or redundant prepuce. To reflect sexual maturation, the participants were divided into three groups based on age: <10 years (pre-puberty, n = 3), 10–20 years (puberty, n = 17), and >20 years (post-puberty, n = 12). The samples were fixed in 4% buffered formaldehyde and routinely processed for paraffin embedding. These materials were obtained from our laboratory (Registro Nacional de Biobancos, Sección Colecciones, Ref. C-0001627). This study was approved by the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. Celm, Past: Proyecto 266/18), and tissue samples were obtained in accordance with Spanish law (RD 1301/2006; Ley 14/2007; DR 1716/2011; Orden ECC 1414/2013).

2.2 | Immunohistochemistry

Deparaffinized and rehydrated sections were processed for indirect immunohistochemistry using a Leica Bond Polymer Refine Detection Kit (Leica Biosystems™) following the manufacturer's instructions. Immunohistochemistry was performed to explore the expression of antigens related to axons (neurofilament proteins, neuron specific enolase [NSE]), corpuscular glial cells (S100 protein [S100P], glial-fibrillary acidic protein [GFAP], vimentin), the endoneurium (CD34 antigen), the perineurium (glucose-transporter 1 [GLUT1]), and the mechanoprotein PIEZO2. Table 1 summarizes the primary antibodies used in this study. Indirect immunohistochemistry included several negative and positive controls, as well as internal and external controls.

2.3 | Double immunofluorescence staining

Double immunofluorescence staining was performed to investigate PIEZO2, S100P, and NSE expression. Non-specific binding was reduced in the deparaffinized and rehydrated sections through incubation for 30 min with a solution of 5% bovine serum albumin in Tris-buffered saline (pH 7.4). The sections were then incubated overnight at 4°C in a humid chamber with a 1:1 (v/v) mixture of anti-S100P

TABLE 1 Primary antibodies used in this study

Antigen	Origin	Dilution	Supplier
CD34 (clone QB-END/10)	Mouse	Prediluted	Master Diagnostica[a]
Glial-fibrillary acidic protein (clone G-A−5)	Mouse	1:500	Boehringer Mannheim[b]
GLUT1	Rabbit	0.5 μg/ml	Cell Marque[c]
NSE (clone BBS/NC/VI-H14)	Mouse	1:1000	Dako[d]
PIEZO2[g]	Rabbit	1:200	Sigma-Aldrich[e]
S100 protein (clone 4C4.9)	Mouse	1:1000	Thermo Scientific[f]
S100 protein	Rabbit	1:1000	Dako[d]
Vimentin (clone 3B4)	Mouse	Prediluted	Dako[d]

Abbreviations: ASIC2, acid sensing ion channel subunit 2; GLUT1, glucose transporter 1; NSE, neuron specific enolase; PIEZO2, piezo-type mechanosensitive ion channel component 2.

[a]Granada, Spain.

[b]Indianapolis, Indiana, USA.

[c]Seattle, WA, USA.

[d]Glostrup, Denmark.

[e]Saint Louis, MS, USA.

[f]Freemont, CA, USA.

[g]Amino acid sequence: FEDENKAAVRIMAGDNVEICMNLDAASFSQHNP.

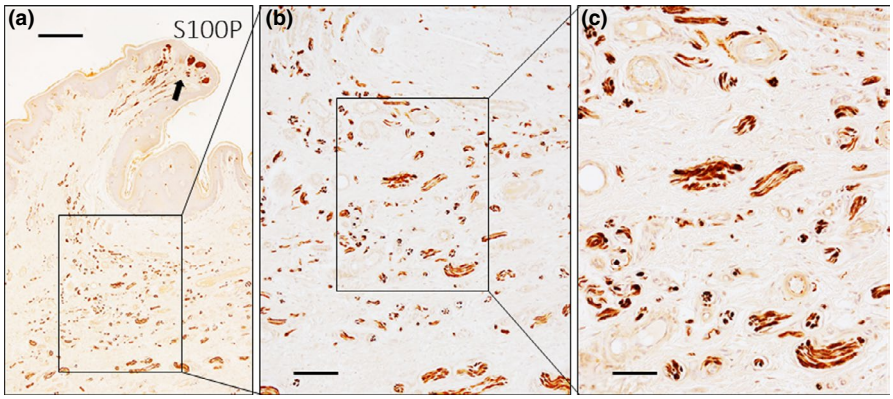

FIGURE 1 Immunohistochemical detection of S100 protein in the human male prepuce. The prepuce contains a dense network of nerve fibers of varying diameters in the deep dermis (image magnification: a → c). Most of the dermal papillae do not show nerve profiles, except for a few Meissner's corpuscles. Arrows in (a). Scale bar: 250 (a), 150 (b), and 80 μm (c) [Colour figure can be viewed at wileyonlinelibrary.com]

and anti-PIEZO2, and anti-NSE and anti-PIEZO2. After rinsing, the sections were incubated for 1 h with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (Serotec™, diluted 1:1000), rinsed again, and incubated for another hour with Cy3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch™, diluted 1:50). Both these steps were performed at room temperature in a dark, humid chamber. The sections were then washed, mounted with Fluoromount Gold (ThermoFisher), and counterstained with 4,6-diamidino-2-phenylindole (10 ng/ml) to label the nuclei. The samples were visualized following triple staining using a Leica DMR-XA automatic fluorescence microscope with Leica Confocal software, version 2.5 (Leica Microsystems, Heidelberg GmbH), and the captured images were processed using ImageJ software, version 1.43 (Master Biophotonics Facility, McMaster University, Ontario, www.macbiophotonics.ca). As controls, representative sections were also processed using the techniques described above, using non-immune rabbit or mouse sera instead of primary antibodies or by omitting the primary antibodies during incubation.

2.4 | Quantitative analyses

Quantitative analyses were performed to determine the density of Meissner's corpuscles using the technique proposed by Verendeev et al., (2015), which has been described in detail in a previous study (García-Piqueras et al., 2019). The Meissner index was determined using the technique proposed by Bhat et al., (2008). The densities of other sensory corpuscles were not calculated because of their infrequent occurrence and irregular distribution in the dermis. Briefly, S100P immunohistochemistry was performed to identify

Meissner's corpuscles in five sections from each skin sample that were obtained from locations 200 μm apart. The sections were scanned using an SCN400F scanner (Leica Biosystems™), and the scans were computed using SlidePath Gateway LAN software (Leica, Leica Biosystems™). Subsequently, Meissner's corpuscles were identified and counted by two independent observers (YG-M and JAV). The average counts were corrected using the Abercrombie formula. The epidermal length (mm) of each section, and the average epidermal length was multiplied by the section thickness (mm) to calculate the surface area (mm^2). Finally, the average number of Meissner's corpuscles (N) was divided by the surface area (mm^2) to calculate the density of Meissner's corpuscles per square millimeter of skin (number of Meissner's corpuscles/mm^2). Subsequently, the average density was calculated from the individual densities for each pre-established age group.

To investigate the relationship between Meissner's corpuscles and dermal papillae, the measurements were standardized according to the length of skin analyzed to compare between the groups. Significant differences among the three pre-established age groups were assessed using the Kruskal–Wallis H test, and p values <0.05, were considered statistically significant.

3 | RESULTS

The prepuce samples were richly innervated by nerve fibers of various diameters that formed a dense network in the reticular dermis. These fibers formed perivascular plexuses, terminated as free nerve endings, and innervated sensory corpuscles of various morphotypes (Figure 1). Human male prepuce is richly innervated by nerve fibers of various diameters that form a dense network in the reticular dermis. These fibers form perivascular plexuses, terminate as free nerve endings, and innervate sensory corpuscles of various morphotypes (Figure 1). Nerve profiles were not observed in most parts of the papillary dermis, except where Meissner's corpuscles were found. Epidermal free nerve endings were scarcely observed (Figure 1a).

3.1 | Meissner's corpuscles are the most common sensory corpuscles in the prepuce

The sensory corpuscles located immediately beneath the epithelium were typical or ovoid Meissner's corpuscles. They displayed a notable tendency to aggregate in the cutaneous folds; their density was high in this region, while they were scarcely present in adjacent areas that were flat or depressed (Figure 2). They showed various morphologies and sizes, were located either in the dermal papillae or the superficial dermis, and displayed the characteristic properties of Meissner's corpuscles (Figure 3a–e). Nevertheless, we also found other corpuscles that were rounded, glomeruloid, or lobulated and were located deep in the dermis; they may have been genital bodies (Figure 2f–h). The morphology, size, and dermal topography of the Meissner's corpuscles were not correlated with age.

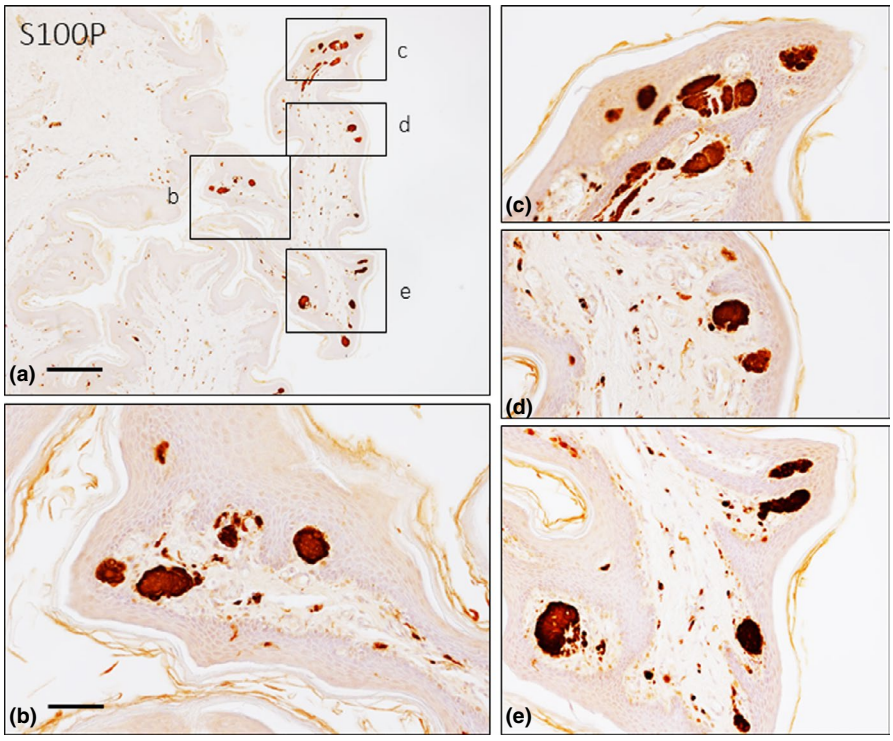

FIGURE 2 Immunohistochemical detection of S100 protein in the sensory corpuscles of the human prepuce. Clusters of Meissner's corpuscles are seen in the cutaneous folds. In contrast, deeper portions of these folds do not show Meissner's corpuscles (a). Images (b)–(e) show details of the Meissner's corpuscles at epidermal excrescences. Scale bar: 250 (a) and 50 μm (b–e) [Colour figure can be viewed at wileyonlinelibrary.com]

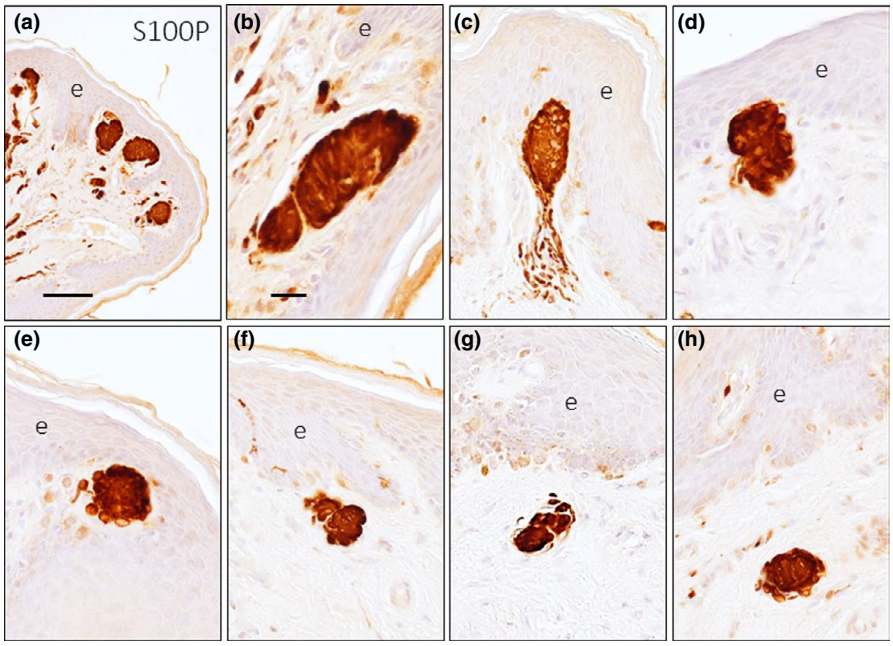

FIGURE 3 Typical Meissner's corpuscles were observed in the dermal papillae (a–e) whereas genital bodies showing rounded, glomeruloid, or lobulated shapes were observed at deeper levels in the dermis (f–h). e: epidermis. Scale bar: 70 (a) and 20 μm (b–h) [Colour figure can be viewed at wileyonlinelibrary.com]

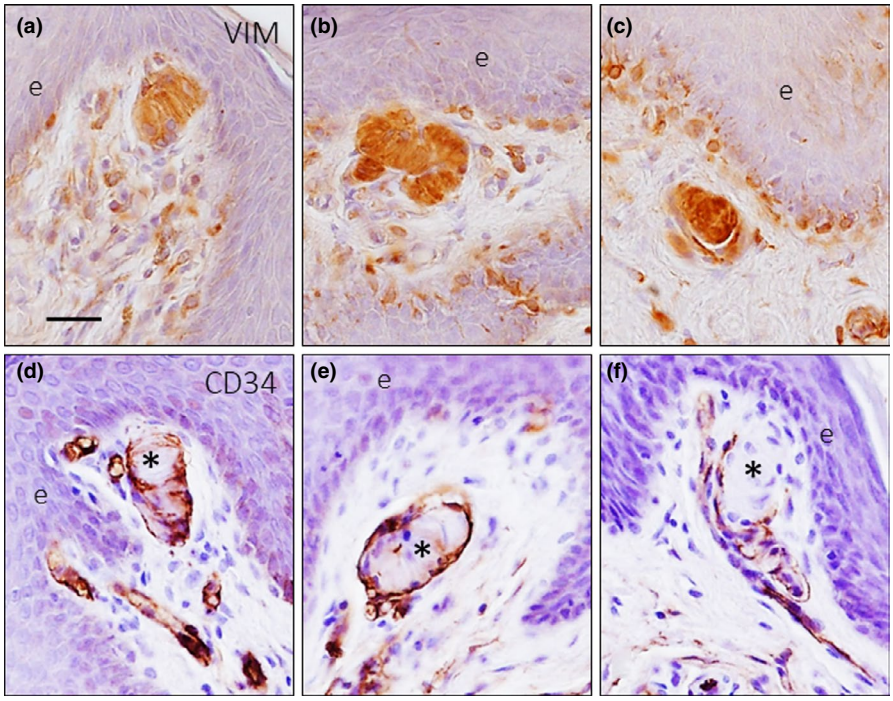

FIGURE 4 The cytoplasm of peripheral glial cells in Meissner's corpuscles and genital bodies displays vimentin immunoreactivity (a–c). Moreover, most of the preputial Meissner's corpuscles and genital bodies are surrounded by a CD34-positive capsule of endoneurial origin (d–f; asterisk: Meissner's corpuscle). e: epidermis. Scale bar: 40 μm (a–f). [Colour figure can be viewed at wileyonlinelibrary.com]

Generally, lamellar cells of sensory corpuscles display strong immunoreactivity for S100P regardless of their anatomical location (Figure 3). As seen in digital Meissner's corpuscles, the preputial Meissner's corpuscles and genital bodies also had lamellar cells displaying vimentin immunoreactivity (Figure 4a–c). No immunoreactivity for GFAP was observed (data not shown). In contrast, approximately 75% of the preputial Meissner's corpuscles were surrounded, partially or completely, by a thin CD34-positive capsule that was

related to endoneurium-derived fibroblasts (Figure 4e,f). Positive GLUT1 immunoreactivity was not detected in the structures resembling sensory corpuscles.

The density and average index of Meissner's corpuscles in the analyzed samples were 8.3 ± 4.1 and 0.14 ± 0.001, respectively. The density of Meissner's corpuscles in participants aged <10 years, between 10 and 20 years, and >20 years was 3.0 ± 1.1, 11.3 ± 4.1, and 10.8 ± 3.7, respectively (Figure 5a). The average Meissner index was 0.02 ± 0.001 in the first decade of life, 0.21 ± 0.01 in the second decade, and 0.28 ± 0.01 in subsequent decades (Figure 5b). Significant differences ($p < 0.001$) were found in both parameters between participants in the pre-puberty group and those in the other two groups, while no significant differences in density or index of Meissner's corpuscles were found between the puberty and post-puberty groups.

3.2 | Meissner's corpuscles of the prepuce show PIEZO2 expression

In this study, intense PIEZO2 immunoreactivity was observed in the axons of Meissner's corpuscles (Figure 6a–d), as well as in scattered Merkel cells (Figure 6e,f). The pattern of axonal PIEZO2 immunostaining was punctate and did not show the entire axonal profile. Double immunolabeling was performed to confirm the localization of PIEZO2 within Meissner's corpuscles. It revealed that PIEZO2 did not co-localize with S100P (thus excluding glial localization) and matched the distribution of neuron-specific enolase well (thus confirming axonal localization, Figure 7).

3.3 | Other morphotypes of sensory corpuscles in the male prepuce

In addition to the characteristic Meissner's corpuscles and Meissner-like sensory corpuscles described above, a few other morphotypes of sensory corpuscles were identified. As a rule, their density was very low, and they showed an irregular morphology. Those that were located in the superficial papillary dermis might have been Krause or Krause-like corpuscles. These corpuscles were easily observable and larger than the previously described superficially located corpuscles (Figure 8). They were generally composed of two glomeruloid elements separated by a hypocellular tissue (Figure 8a), and a capsule surrounded all the neural elements (Figure 8b,c). A few deeply located corpuscles showed the characteristic morphology of Ruffini corpuscles (Figure 9a) and Ruffini-like corpuscles (Figure 9b,c). They were elongated, associated with Schwann-like cells, and surrounded by a nearly developed capsule. In addition, we found a great variety of morphologically undetermined small sensory corpuscles. They were round; composed of several loosely disposed, Schwann-derived cells; and occasionally showed a longitudinal or lamellar structure (Figure 9e–h).

4 | DISCUSSION

Human male prepuce is involved in sensation of diverse mechanical and non-mechanical stimuli. It was found to contain sensory formations of various morphotypes, especially Meissner's corpuscles in the dermal papillae; rounded, deeply located corpuscles that correspond to the genital bodies; and a few sensory corpuscles (Ruffini and Krause corpuscles) of other morphotypes.

The most striking feature of preputial skin is the low degree of innervation of the papillary dermis. While previous studies have not investigated this paucity of innervation, it could be related to site-specific variations within the prepuce sample studied (Martín-Alguacil et al., 2015). This is supported by the fact that there is notable innervation of areas with sensory corpuscles. In our previous study on genital skin (Feito et al., 2018a), we found that Meissner's corpuscles were usually located in the skin folds; this was also the case in the prepuce (Figure 1). These folds probably correspond to the ridged bands and are similar to the vulvar folds; both of these are rich in corpuscles (Cold & Taylor, 1999; Feito et al., 2018a; Sorrells et al., 2007).

Meissner's corpuscles were observed in abundance in the prepuce. They were not morphologically different from those in digital skin, thus being termed Meissner's corpuscles and not Meissner-like corpuscles, which are found in the vulvar labia minora (Feito et al.,

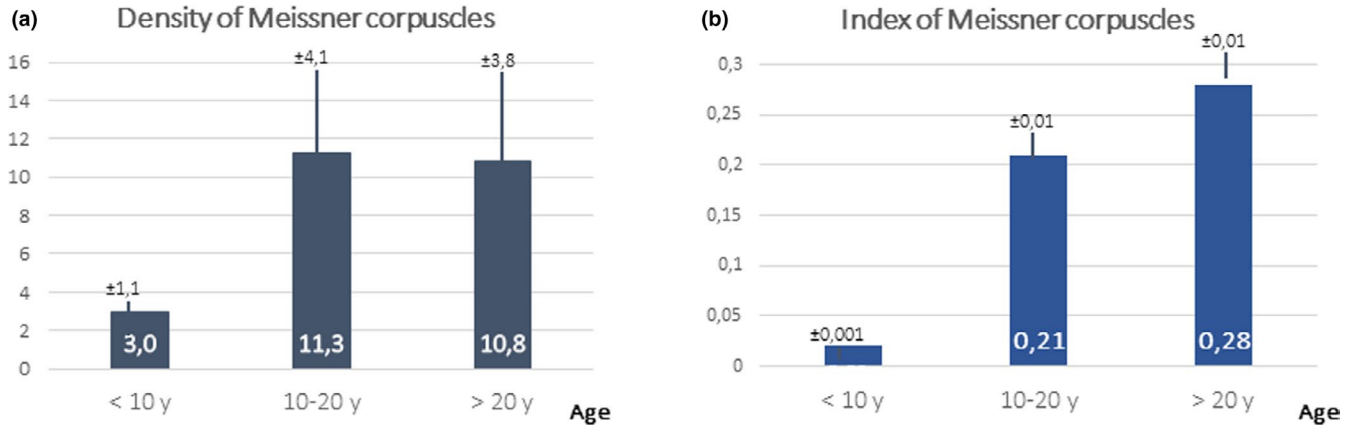

FIGURE 5 Results of the quantitative study [Colour figure can be viewed at wileyonlinelibrary.com]

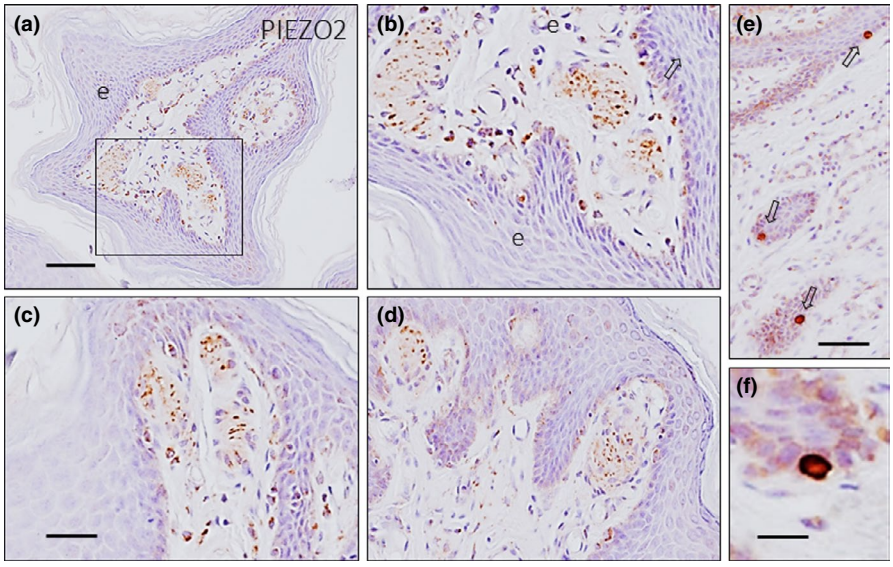

FIGURE 6 Piezo-type mechanosensitive ion channel component 2 (PIEZO2) immunoreactivity of the Merkel cells (e–h) and the axon supplying the Meissner's corpuscles (a–d). In the latter, the pattern of distribution of PIEZO2 is not consistent throughout the axon but is dotted (e–h; e: magnified image of d). Scale bar: 120 μm (a, d, f–h), 30 μm (b, c, e) [Colour figure can be viewed at wileyonlinelibrary.com]

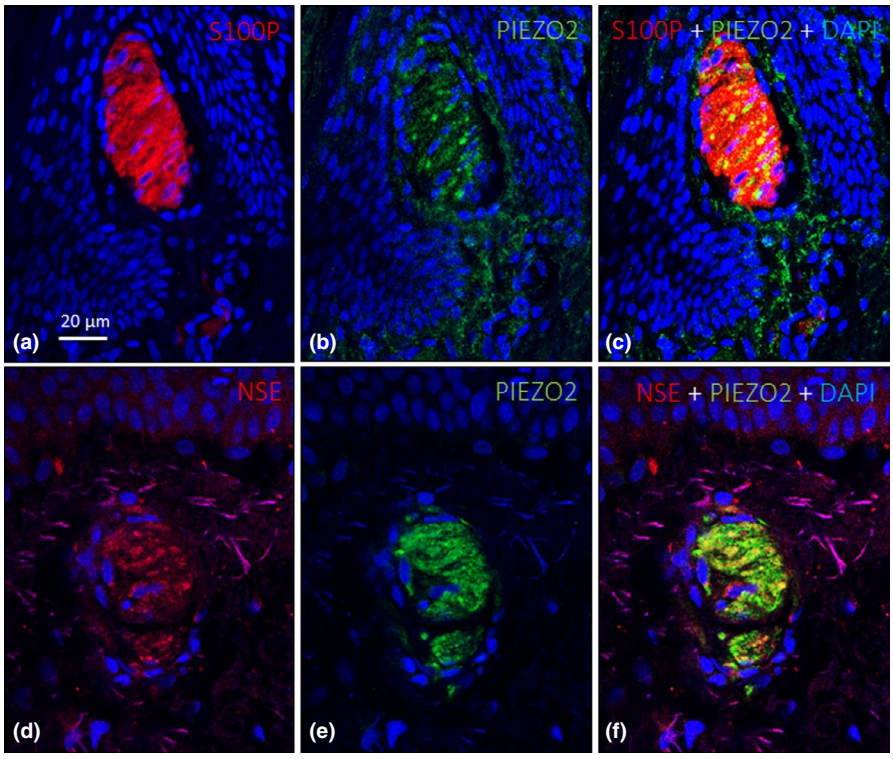

FIGURE 7 Double immunofluorescence for piezo-type mechanosensitive ion channel component 2 (PIEZO2; green fluorescence; b, c, e, and f) and either S100P (red fluorescence; a and c) or neuron specific enolase (NSE; red fluorescence; d and f) confirms the presence of PIEZO2 in the axon (co-localization with NSE, yellow merge; f) and the absence of PIEZO2 in the lamellar cells (no co-localization with S100P, no merge; c). Sections were counterstained with 4,6-diamidino-2-phenylindole to ascertain structural details. Objective: 63×/1.40 oil, pinhole: 1.37, *XY* resolution: 139.4 nm, and *Z* resolution 235.8 nm. Scale bar: 20 μm [Colour figure can be viewed at wileyonlinelibrary.com]

2018a). These results are consistent with those of previous studies (Cold & Taylor, 1999; Halata & Munger, 1986; Martin-Alguacil et al., 2015; Taylor et al., 1996). Furthermore, preputial Meissner's corpuscles and genital bodies share the immunohistochemical characteristics of their digital counterparts, and most of them have a thin capsule of endoneurial origin (Cobo et al., 2021; García-Piqueras

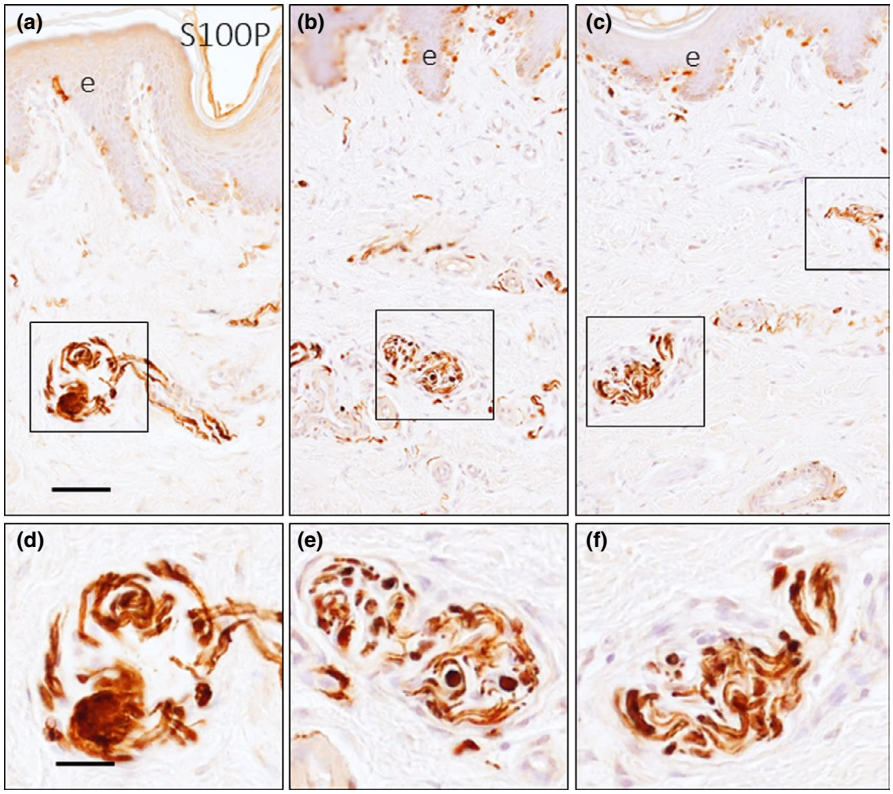

FIGURE 8 The deep dermis contains various morphotypes of sensory corpuscles, including Krause or Krause-like corpuscles. They are observed on immunohistochemistry for S100P. Compared with the Meissner's corpuscles, these are bigger, have a more irregular morphology, and are located deeper in the dermis (a–c). Constitutively, the two neural glomeruloid elements (a) are surrounded by a capsule (b and c). d–f: image magnifications of a–c, respectively. e: epidermis. Scale bar: 50 (a–c) and 20 μm (d–f) [Colour figure can be viewed at wileyonlinelibrary.com]

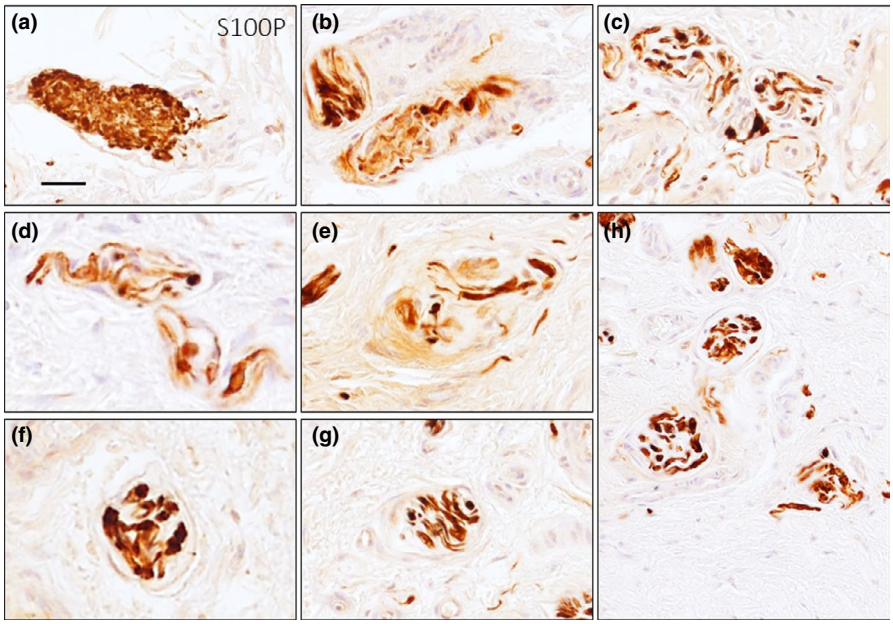

FIGURE 9 S100P immunostaining reveals different morphotypes of sensory corpuscles identified respectively as Ruffini (a) and Ruffini-like corpuscles (b–c). Moreover, other unidentifiable sensory corpuscles with undetermined morphology and loose structures were also observed (d–h). Scale bar: 60 μm (a–h) [Colour figure can be viewed at wileyonlinelibrary.com]

et al., 2020; Vega et al., 2009). Interestingly, the density of Meissner's corpuscles increased during adolescence and in early adulthood. This might have been related to the completion of maturation of the Meissner's corpuscles, leading to better identification of these corpuscles (see Feito et al., 2018b). Alternatively, it could be related to pubertal changes in the preputial skin. This is supported by the fact that the labia minora shows widespread changes in three defined phases that correspond to the prepubertal, pubertal, and postpubertal stages (Feito et al., 2018a).

Genital end bulbs are corpuscular structures that are closely related to Meissner's corpuscles. They differ from Meissner's corpuscles in morphology and are located in the dermis (Halata & Munger, 1986). These sensory structures are more deeply located than Meissner's corpuscles and share their immunohistochemical profiles. Ruffini corpuscles are rarely observed in the human prepuce. Some authors have identified them as secondary structures involved in preputial innervation (Halata & Munger, 1986), while others did not observe them (Cold & Taylor, 1999; Martin-Alguacil et al., 2015). Here, we observed typical Ruffini corpuscles and a few Ruffini-like corpuscles. Additionally, we observed rounded sensory corpuscles that might have been Krause corpuscles. Krause corpuscles have been previously identified in the human prepuce (Martin-Alguacil et al., 2015). Unlike other studies (Halata & Munger, 1986; Martin-Alguacil et al., 2015), we did not find Pacinian corpuscles in the human prepuce. In any case, sensory corpuscles other than Meissner's corpuscles are scarcely found in the human prepuce.

The most striking finding of this study was the demonstration of PIEZO2 immunoreactivity in Merkel cells and axons of both preputial Meissner's corpuscles and genital end bulbs, which supports the role of these corpuscles as mechanoreceptors. In fact, in cutaneous mechanoreceptors, the conversion of mechanical stimuli into electrical signals (mechanotransduction) involves mechano-gated ion channels, especially PIEZO2 (García-Mesa et al., 2017; García-Piqueras et al., 2019; Ranade et al., 2014), although other proteins could be involved or indirectly participate in it (Alonso-González et al., 2017; Cabo et al., 2015; Calavia et al., 2010). Here, we observed that the axons of the preputial sensory corpuscles displayed PIEZO2 immunoreactivity similar to that of digital Meissner's corpuscles (García-Mesa et al., 2017; García-Piqueras et al., 2019).

Preputial Merkel cells (Cold & Taylor, 1999; Halata & Munger, 1986) were occasionally observed in our study. They displayed PIEZO2 immunoreactivity. They were not observed in a previous study (Martín-Alguacil et al., 2015). We believe that this is the first time that the presence of mechanoproteins, particularly PIEZO2, in the preputial sensory corpuscles has been reported. These findings are of interest because mechanical stimulation of the male prepuce plays a role in sexual arousal, and PIEZO2 is critical for mechanotransduction (Wu et al., 2017).

Nevertheless, the role of the prepuce in sexual pleasure and orgasm is a matter of debate (Jenkins, 2014; Morris et al., 2019; Poland, 1990). Most of the information on this topic comes from clinical data of individuals undergoing circumcision for religious, social, medical, or preventive purposes (Morris et al., 2013, 2014, 2016, 2019). A recent meta-analysis failed to demonstrate significant clinical alterations associated with circumcision (Tian et al., 2013). Some authors have reported that circumcision decreases penile sensitivity (Bronselaer et al., 2013; Sorrells et al., 2007), while others have failed to demonstrate any difference (Bleustein et al., 2005; Payne et al., 2007) or have disputed the results (Hegarty, 2013; Morris et al., 2013). It is possible that the prepuce has been proposed to play a role in sexual function because of the belief that it must have a particular role (Martin-Alguacil et al., 2015). It has been demonstrated that mechanical stimulation of the prepuce leads to activation of the external urethral sphincter in rats (Juárez et al., 2016). Although the effects of circumcision on penile sensitivity and sexual arousal are probably minor, the prepuce is the most sensitive area of the penis (Bossio et al., 2016), which is indicated by the rich innervation observed on microscopy in this study.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to the publication of this paper.

AUTHOR CONTRIBUTIONS

YG-M, RC, and JM-C conducted the experiments. JF and OG-S collected and processed the material in compliance with the ethical guidelines and performed some parts of the experiments. YG-M and JG-P quantified and evaluated the data. IS, JF, and JAV designed the study, analyzed the data, and wrote the manuscript.

ETHICAL STATEMENT

This study was approved by the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. CElm, PAst: Proyecto, 266/18) Informed consent was obtained from either the patient or his parent/legal tutor, and all of these materials used in the study were obtained in accordance with Spanish law (RD 1301/2006; Ley 14/2007; DR 1716/2011; Orden ECC 1414/2013).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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3.6. Resultados – Manuscrito 6

Journal of Clinical Medicine

Yolanda García-Mesa, Patricia Cuendias, Abel M. Gago, Jorge Feito, José A. Vega, Olivia García-Suárez.

PIEZO 1 AND PIEZO2 IN THE HUMAN MALE AND FEMALE GENITOURINARY SYSTEMS. An immunohistoclemical study (pendiente publicación)

Journal of
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Article

PIEZO1 AND PIEZO2 IN THE HUMAN MALE AND FEMALE GENITOURINARY SYSTEMS. An immunohistochemical study

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Abstract: PIEZO1 and PIEZO2 are essential proteins of mechanosensing ion channels gated by distension, flow or movement. Thus, they are candidate to be present in the genitourinary system. Here we used immunohistochemistry to analyze the distribution of those proteins in the organs of the male and female human genitourinary tract. In the kidney and urinary tract specific immunoreactivity for PIEZO1 was detected in the epithelium of the proximal and distal renal tubules, collecting tubules, epithelium and muscular of the ureter; no immunoreactivity was detected in the urinary bladder; PIEZO2 it was detected in the same localizations as PIEZO1. Positive PIEZO1 and PIEZO2 cells were detected in the ovary, presumably primordial follicles, testicles, fallopian tubes and prostate. Using double immunohistochemistry we demonstrated that most of the PIEZO immunoreactive cells were epithelial. Present results demonstrate that PIEZO mechanosensor proteins are expressed in human non-nervous tissues and might be involved in different urinary and reproductive functions.

Keywords: mechanosensors; PIEZO1; PIEZO2; urogenital system; human

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1. Introduction

Mechanical biological transducers are proteins (mechanoproteins) responsible for the conversion of mechanical forces into electrical or biochemical signals that allow the cell to respond to environmental changes (Martino et al., 2018). Proteins forming a part of several families and superfamilies of ion channels have been identified as putative mechanoproteins (Jin et al., 2020; Swaminathan and Gloerich, 2021). However the only ion channel family that fulfil the criteria to be regarded as true mechanotransducers are Piezo channels (Murthy et al, 2017; Zang et al., 2019). The family of Piezo ion channels consists of two members, PIEZO1 and PIEZO2. They are stretch-gated multipass transmembrane proteins (Saotome et al., 2018; Zhao et al., 2018; Wang et al., 2019; Fang et al., 2021) necessary for non-selective cationic mechanosensitive channels in mammalian cells directly activated by mechanical forces (Coste et al., 2010; Seyda et al., 2016; Geng et al., 2017).

PIEZO1 and PIEZO2 are wide expressed in both nervous and not-nervous tissues. Focusing on the genitourinary system, PIEZO1 and PIEZO2 mRNA and immunoreactivity was detected in the kidney, urethra, urinary bladder, vagina, prostate glands, seminal

vesicles, and ejaculatory ducts (Martins et al., 2016; Peyronnet et al., 2013; Etem et al., 2018; Dalghi et al., 2019). On the other hand, functional studies suggest that PIEZO1 is involved in the regulation of urinary osmolarity (Martins et al., 2016), whereas PIEZO2 seems to participate in the regulation of urine flow and bladder distention.

As far as we known the distribution of PIEZO1 and PIEZO2 in the human genitourinary system was never investigated. Therefore, the present research was designed to systematically investigate the distribution of those proteins in the human genitourinary system using immunohistochemistry.

2. Materials and Methods

2.1. Materials and treatment of tissues.

Samples of kidney (n = 10), ureter (n = 8), bladder (n = 6), prostate (n = 12), testicle (n = 5, fallopian tubes (n 0= 4), uterus (n = 5), and ovary (n = 3) were obtained from the collection of SINPOS research group, University of Oviedo (Registro Nacional de Biobancos, Sección Colecciones, Ref. C-0001627). They were taken from autopsies, free of urinary and genital diseases. The age range of the patients was 25-50 years. All materials were obtained in compliance with Spanish Laws (RD 1301/2006; Ley 14/2007; DR 1716/2011; Orden ECC 1414/2013) and according to the guidelines of the Declaration of Helsinki II (World Medical Association, 2013). Tissue samples were fixed in 10% buffered formaldehyde, dehydrated and routinely embedded in paraffin. Sections of 7 μm or serial sections were cut onto silane-coated slides and deparaffinized. One section from each tissue was stained with hematoxylin and eosin for morphological evaluation, and those tissues that showed pathological changes were excluded from the study.

2.2. Methods.

Deparaffinized and rehydrated sections were processed for detection of PIEZO1 and PIEZO2 using the EnVision antibody complex detection kit (Dako, Copenhagen, Denmark) following the supplier's instructions. Briefly, endogenous peroxidase activity was inhibited (3% H2O2 for 15 min), and non-specific binding was blocked (10% bovine serum albumin for 20 min). Sections were then incubated overnight at 4 °C with the primary antibodies (see Table 1). Subsequently, the sections were incubated with anti-rabbit EnVision system-labelled polymer (DakoCytomation) for 30 min. Finally, the slides were washed with buffer solution, and the immunoreaction was visualized with diaminobenzidine as a chromogen, washed, dehydrated, and mounted with Entellan (Merck, Dramstadt, Germany). To ascertain structural details, the sections were counterstained with Mayer's haematoxylin.

To accurately identify the structures and cellular types displaying PIEZO1 and PIEZO2 immunoreactivity in some selected tissues, sections were also processed for simultaneous detection of PIEZO1/2 together with β-tubulin (TUB2.1) (cilium marker in fallopian tube and proximal tubules, collecting ducts and primary cilia in kidney), vimentin (marker of Sertoli cells in seminiferous tubes) and S100 (marker for Leyding cells in seminiferous tubes). Non-specific binding was reduced by incubating the sections for 30 min with a solution of 25% calf bovine serum in Tris buffer solution (TBS). The sections were incubated overnight at 4 °C in a humid chamber with a 1:1 v/v mixture of the polyclonal antibody against Piezo2 described above (used diluted 1:100). After rinsing with TBS, the sections were incubated for 1 hour with Alexa fluor 488-conjugated goat anti-rabbit IgG (Serotec, Oxford, UK), diluted 1:1000 in TBS containing 5% mouse serum (Serotec), then rinsed again and incubated for another hour with Cy 3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch, Baltimore, MD, USA) diluted 1:50 in TBS. Both steps

were performed at room temperature in a dark humid chamber. Sections were finally washed, and the cell nuclei stained with DAPI. Triple fluorescence was detected using a Leica DMR-XA automatic fluorescence microscope (Microscopía Fotónica y Proceso de Imagen, Servicios Científico-Técnicos, Universidad de Oviedo) coupled with Leica CONFOCAL Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany), and the images captured were processed using IMAGEJ software version 1.43g [Master Biophotonics Facility, McMaster University Ontario (www.macbiophotonics.ca)].

For control purposes, representative sections were processed in the same way as described but using non-immune rabbit or mouse sera instead of the primary antibodies or omitting the primary antibodies in the incubation. Furthermore, when available, additional controls were carried out using specifically preabsorbed antisera. Under these conditions, no positive immunostaining was observed (data not shown).

Table 1. Antibodies used in the study

Antibody / clon3	Catalog number	Host	Dilution	Source
PIEZO2	A105249	Rabbit	1:200	Sigma Aldrich (Madrid, Spain)
PIEZO1	PA5-72974	Rabbit	1:200	Invitrogen (California, USA)
S100 / (4C4.9)	GTX24066	Mouse	1:2000	Genetex (Irvine, CA, USA)
Vimentin / (V9)	IS630	Mouse	Prediluted	Dako (Glostrup, Denmark)
Actin / (HHF35)	IS700	Mouse	Prediluted	Dako (Glostrup, Denmark)
β-tubulin / (TUB2.1)	T4026	Mouse	1:300	Sigma (St Louis, MS, USA)
Chromogranin A / (LK2H10)		Mouse	1:200	Boeringer (Mannheim,Germany)

3. Results

3.1. Urinary system

In the urinary system, in the kidney, PIEZO1 and PIEZO2 immunostaining was detected in the epithelium of the tubules but not with the same intensity in all of them: the intensity is higher to PIEZO2 (++ vs. +). In contrast, none of the PIEZO staining was presented in the cells of the glomeruli. To determine the type of tubule which were positive for PIEZO2, double immunofluorescence with PIEZO and β-tubulin is performed (Figure 1). Tubulin showed strongest expression (bright red) in the proximal tubules and collecting ducts due to the presence of primary cilia (Saraga-Babic et al., 2012). Merged view of a+b+dapi demonstrated that PIEZO2 was expressed strongly by proximal tubules and collecting duct and weakly expressed in distal tubule. The same occurs with PIEZO1, but the immunostaining is lower. In the ureter, the urothelium was positive whereas elastic fibers and connective tissue within the lamina propria were negative, and once again, the immunoreaction is higher for PIEZO2. Finally, the bladder was negative for both proteins (Fig. 2).

3.2. Male genital system

In the male reproductive system, selective immunostaining of PIEZO was found in cells of the prostate as in both acinus and ducts (PIEZO2 is more intensive that PIEZO1). Confocal microscopy permitted us to demonstrate that PIEZO positive cells of prostate were enolase positive, in fact mergence image showed yellow colour where was colocalization of both proteins, this date indicated that PIEZO positive cells were endocrine cells (Fig. 3). In the testis, seminiferous tube are negative for PIEZO proteins,

however some cells are positive for PIEZOs in the interstice, using immunohistochemistry simple (Fig. 4).

Table 2. Immunolocalization of PIEZO1 and PIEZO2 in the urinary system

Urinary system	Kidney	Glomeruli	-	-
		Tubular epithelium	-	-
		Proximal/distal	++	+
		Collecting	++	+
	Ureter	Epithelium	+++	+++
		Muscle layer	++	++
	Bladder	Epithelium	-	-
		Muscle layer	-	-

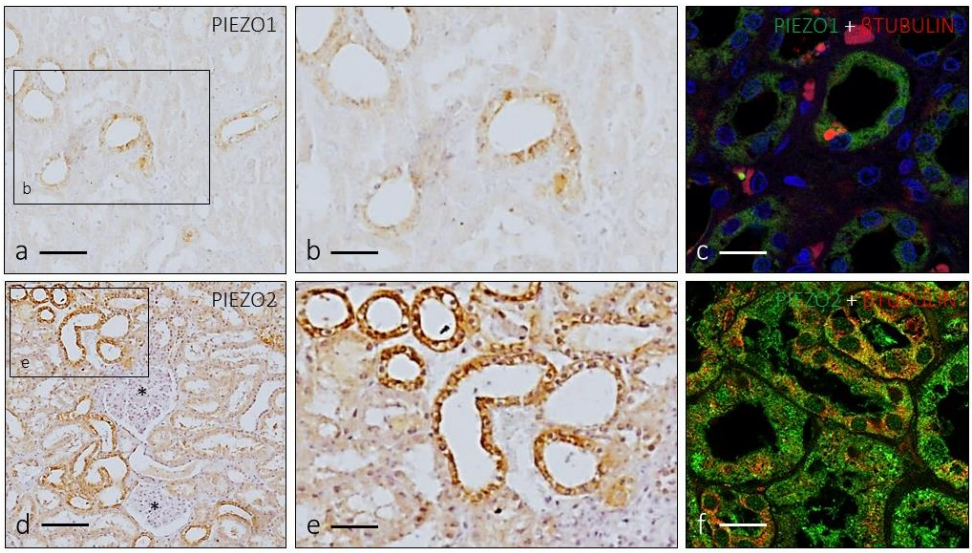

Figure 1. Immunohisrochemical detection of PIEZO1 and PIEZO2 in the human kidney. Scale bar = 50 μm.

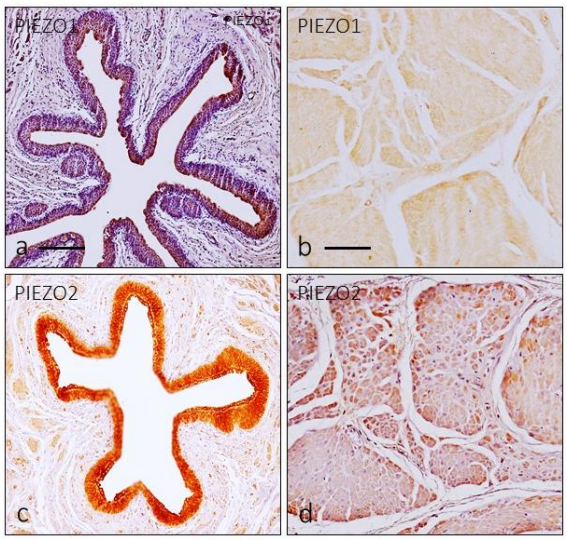

Figure 2. Immunohisrochemical detection of PIEZO1 and PIEZO2 in the human ureter and urinary bladder. Scale bar = 50 μm.

Double immunofluorescence confirmed these results, thus no inflorescence for PIEZOs was detected in seminiferous tube whereas Sertoli cells were positive for

vimentin (marker for Sertoli cells), obviously no colocalization of both markers was detected. In relation to interstice, double staining with PIEZO2 and S100 protein (marker for Leyding cells) demonstrated that Leyding cells were positive for this protein, but not to PIEZO1. The immunoreaction pattern was diffuse in the cellular cytoplasm (Fig. 3).

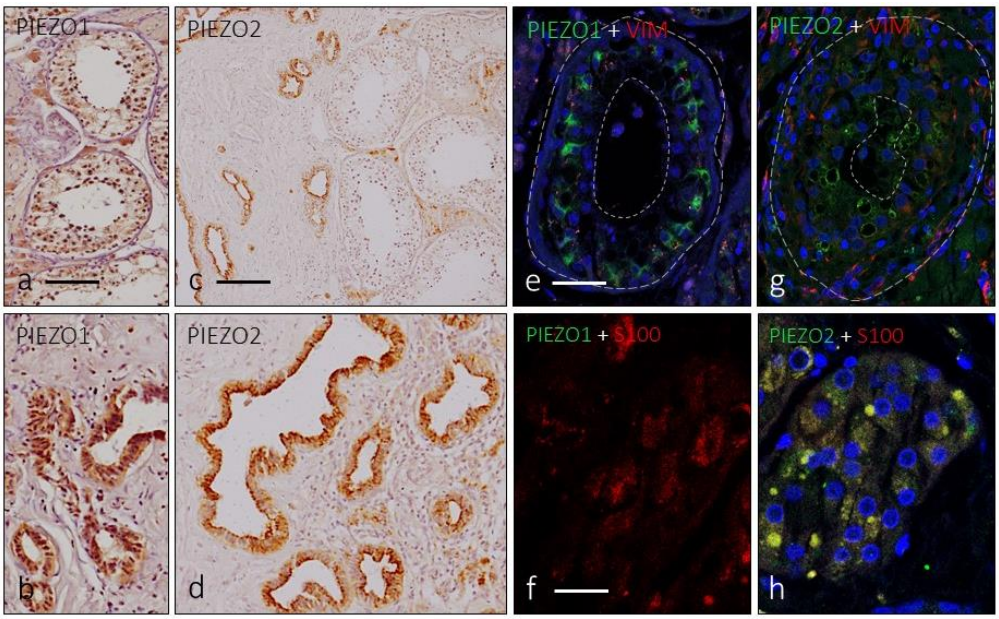

Figure 3. Immunohisrochemical detection of PIEZO1 and PIEZO2 in the human testicle and epididime. Scale bar = 50 μm.

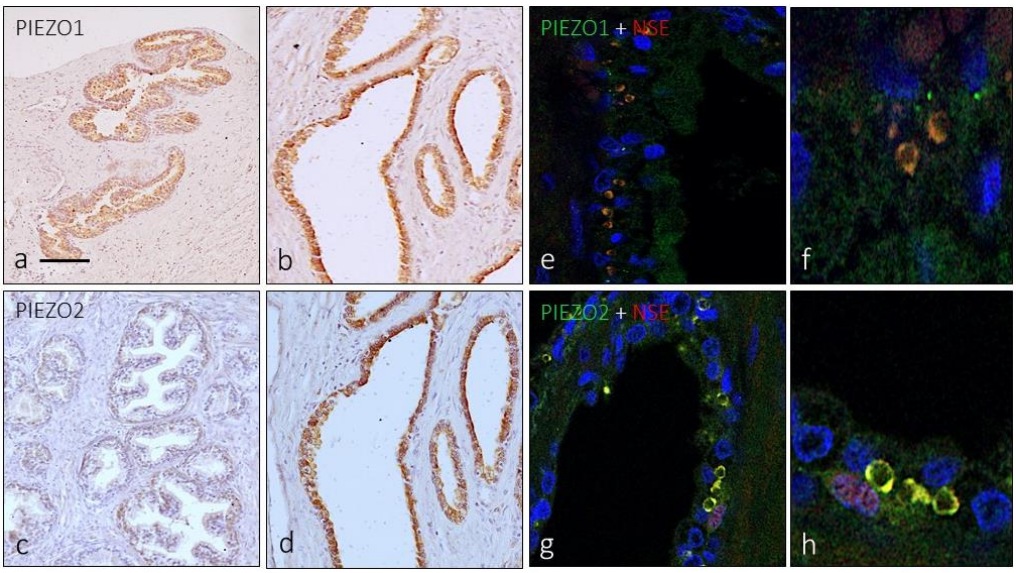

Figure 4. Immunohisrochemical detection of PIEZO1 and PIEZO2 in the human prostate. Scale bar = 50 μm.

3.2. Female genital system

In the female reproductive system, in the ovarian, follicles were negative and epithelial inclusion glands of the stroma were positive for PIEZOs. But oocytes only are positive for PIEZO1. Respect to the uterus, PIEZO2 was found in the epithelium, while PIEZO1 was negative. In the fallopian tubes, the positive immunoreaction was restricted to tubal epithelial cells, in fact some of them showed a strong positive staining for PIEZO2,

while PIEZO1 presents more diffuse immunoreaction, whereas stroma with muscular layers were negative for both.

Table 3. Inmunolocalization of PIEZO1 and PIEZO2 in the male and female genital system

	Organ /Tissue	Cell Type		
Male genital system	Testis	Sertoli cells	+++	++
		Spermatogenic cells	-	-
		Leyding cells	+	-
	Prostate	Acinus	++	++
		Ductus	++	++
Female genital system	Ovary	Oocytes	-	++
		Granulose cells	-	++
		theca cells	-	-
		stroma	-	-
		surface epithelium	+++	++
	Fallopian tube	Epithelium		
		Ciliated cells	+++	+
		Secretory cells	-	-
		Intercalated cells (PEG)	-	-
		Stroma	-	-
	Uterus	Endometrium Epithelium	++	-
		Endometrium stroma	-	-
		Miometrium	-	-

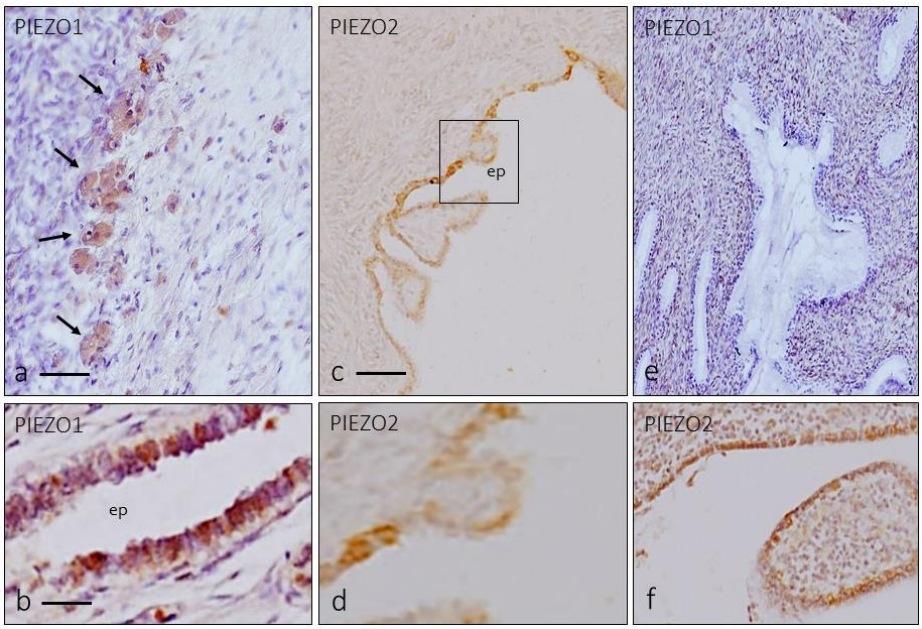

Figure 5. Immunohisrochemical detection of PIEZO1 and PIEZO2 in the human ovary and uterus. Scale bar = 50 μm.

In the tube epithelium there are two cellular types: ciliated and excretory cells. To determine the subset of tubal epithelial cells were positive for PIEZOs, double immunofluorescence associated with confocal microscopy using β-tubulin (cilium marker) and PIEZOs (Hua & Ferland, 2017) was performed, demonstrating that only ciliated cells were positive for PIEZOs.

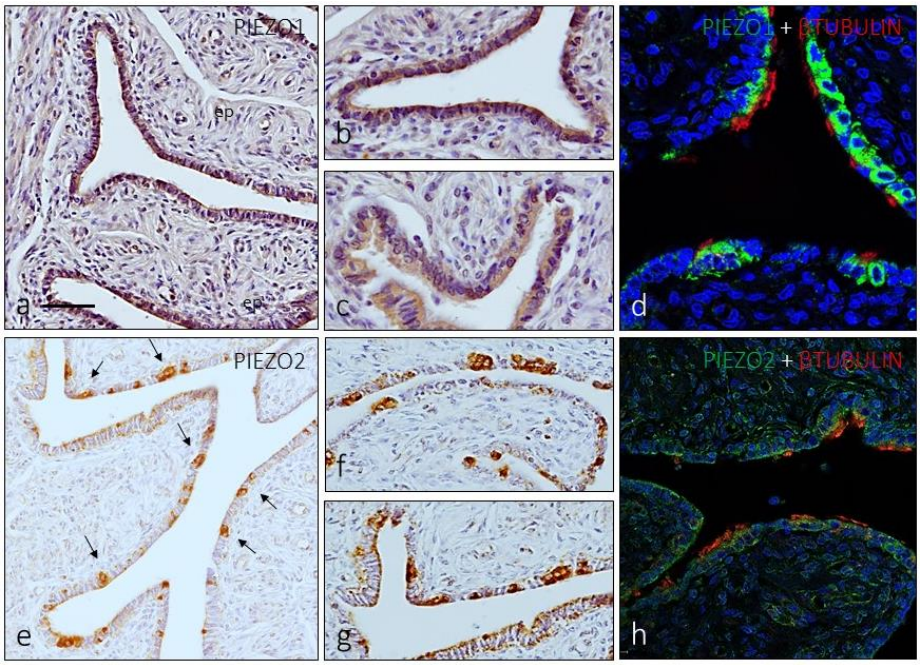

Figure 6. Immunohisrochemical detection of PIEZO1 and PIEZO2 in the human fallopian tube. Scale bar = 50 μm.

4. Discussion

In 2010, the PIEZO family, made up of two proteins, PIEZO1 and PIEZO2, were identified as the great unknowns capable of transducing a mechanical stimulus into electrical signals, in numerous cell types. In recent years, it has been possible to attribute mechanosensitive functions such as proprioception, touch, and regulation of vascular and urinary flow, among many others (Coste et al., 2010; Seyda et al., 2016; Geng et al., 2017). Since their discovery as mechanosensory transduction molecules, studies of PIEZO proteins have revealed many mechano-associated biologic processes such as touch, pain, proprioception and hearing, flow sensing in the kidney, lung inflation, regulation of vascular tone, and muscle and tendon stretch (Nilius, 2010; Coste et al., 2010). However, to date expression, distribution, and localization of PIEZO1 and PIEZO2 protein channel in wide human collection of tissues has not been reported. The immediate goal of our analysis was to use immunohistochemical approach to perform a comprehensive analysis of the expression and localization of PIEZO1 and PIEZO2 in human genito-urinary system. Some of our results have already been confirmed by other investigators. Identification of PIEZO1/2-expressing cell types was the most important consequence of this study. Therefore, we focus our discussion on several organs/tissues that showed peculiar ion channel immunolocalization.

4.1. Urinary system

The proper function of the organs that make up the urinary tract depends on their ability to sense and respond to mechanical forces, and PIEZO´s family is a possible candidate mechano-gated channels implicated in this function. At present, there is a paucity of information about the expression and localization of PIEZO in the genitourinary tract. PIEZO transcripts are detected by quantitive RT-PCR in kidney and bladder (Coste et al., 2010, Etem et al 2018), PIEZO2 protein have only been found in bladder urothelium (Etem et al., 2018) and PIEZO1 immunoreactivity was found in

urethra, bladder, vagina, prostate glands, seminal vesicles, and ejaculatory ducts, especially at epithelial level, and in the cortex and tubules of kidney, but only in mouse (Dalghi et al., 2019). Our study expands on these earlier studies by documenting the sites of PIEZO protein expression in the urinary system in human. In the kidney we found PIEZO2 in the collecting and proximal ducts, as demostrated double inmunostaining with α-tubulin (using to detect the brush border of collecting and proximal ducts) and the same occurs with PIEZO1, but the intensity is lower. Moreover, we detected PIEZO1 and PIEZO2 protein in the urothelium of ureter, this is consistent with PIEZO protein detection in human bladder urothelium (Etem et al., 2018; Dalghi et al., 2019).

The urothelium is considering as sensory tissue that responds to mechanical stress, in fact, numerous TRP channels have been identifiy in the urothelium (Merrill et al., 2016), and it has been reported PIEZO1 expression in the principal cells of the collecting duct (Martins et al., 2016). Our study further indicated that human kidney and ureter are also sites of PIEZO1 and PIEZO2 expression and may similarly use PIEZO as a mecanosensor required for mechanosensation in these tissues, indeed PIEZO has been relacionated with urine flow sensing in the kidney and detection of intraluminal pressure changes (Coste et al., 2010; Nilius, 2010).

4.2. Genital system

Our study further indicates that genital organs in both sexs, like prostate glands, seminiferous tube, ovary, fallopian tube, uterus…. are also sites of PIEZO expression and it must be underlined that this protein inmunostaining always was in epithelial cells. In the case of the male genital tract, the localization of PIEZO1 and PIEZO2 were in both acinis and ducts in prostate whereas in the testis PIEZO2 was situated in Leyding cells as we demostrated using double inmunofluorescence with S100 (a marker for Leyding cells) (Amselgruber et al., 1994). The same occurs with PIEZO1, but the immunoreaction is lower. Respect to female genital system uterus and fallopian tube epithelium was positive for PIEZO1 and PIEZO2 proteins, concretely we demonstrated that ciliates fallopian cells are PIEZO positive. Any aberrations in the normal functioning of fallopian tube epithelium may lead to ectopic pregnancy, whereby the embryo is erroneously implanted in the fallopian tube rather than in the uterus (Woodruff and Julien, 1969).

A fact that draws our attention, despite the fact that immunostaining is almost always similar for PIEZO1 and PIEZO2, is that oocytes exclusively express PIEZO1. This coincides with a study published last year, which states that PIEZO1 acts in different reproductive tissues to promote ovulation and proper fertilization, but this study has only been done previously in Caenorhabditis elegans (Bai et al., 2020).

We believe it is likely that PIEZO will serve as a general mechanosensory in the genitourinary tract, where we are identified multiple sites of PIEZO1 and PIEZO2 expression associated with numerous tissues, it should now be possible to establish whether PIEZO channels are physiologically relevant in these tissues although future definitive functional experiments need to be performed.

To the light of our results, immunohistochemistry technique has permitted us to determine that PIEZO positive cells are in contact with flow of luminal contents and can respond to luminal forces, such as secretion-driven volume expansion and shear stress. This progress will lead to better understanding of PIEZO contribution to cellular and tissular physiology, although follow-up studies using molecular biological tools and conditional knockout models will provide a better understanding regarding the gating and the physiological role of PIEZO channels in various species and tissues in different sensory and non-sensory tissues.

PIEZO2 was originally associated to touch transduction, proprioception, hearing and mechanical pain (Coste et al., 2010). Consistently PIEZO2 was found in mechanical cutaneous sensory afferents and associated cells like Merkel cells (Coste et al., 2010; Ikea

et al., 2014; Maksimovic et al., 2014; Ranade et al., 2014; Woo et al. 2015b; Wu et al., 2016; García-Mesa et al., 2017; Shin et al., 2019). Further studies, however, demonstrated that PIEZO2 is not restricted to the nervous system but it is expressed in a wide range of non-nervous tissues including kidney, bladder, lung, colon, skin, blood vessels, articular cartilage and bone (Coste et al., 2010; Ikeda and Gu 2014; Lee et al., 2014; Woo et al., 2014; Nonomura et al., 2016; García-Mesa et al., 2017; Dickson, 2018; Etem et al., 2018; Huang et al.,2019).

In humans carrying mutations in PIEZO2 show a selective loss of touch perception, have profoundly decreased proprioception, and display complex syndromes that involve joints, ocular muscles, and bone development (McMillin et al., 2014; Alisch et al., 2016; Chesler et al., 2016; Mahmud et al., 2016; Alper, 2017; Masingue et al., 2019).

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3.7. Resultados – Publicación 7

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Involvement of Cutaneous Sensory Corpuscles in Non-Painful and Painful Diabetic Neuropathy

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Abstract: Distal diabetic sensorimotor polyneuropathy (DDSP) is the most prevalent form of diabetic neuropathy, and some of the patients develop gradual pain. Specialized sensory structures present in the skin encode different modalities of somatosensitivity such as temperature, touch, and pain. The cutaneous sensory structures responsible for the qualities of mechanosensitivity (fine touch, vibration) are collectively known as cutaneous mechanoreceptors (Meissner corpuscles, Pacinian corpuscles, and Merkel cell–axonal complexes), which results are altered during diabetes. Here, we used immunohistochemistry to analyze the density, localization within the dermis, arrangement of corpuscular components (axons and Schwann-like cells), and expression of putative mechanoproteins (PIEZO2, ASIC2, and TRPV4) in cutaneous mechanoreceptors of subjects suffering clinically diagnosed non-painful and painful distal diabetic sensorimotor polyneuropathy. The number of Meissner corpuscles, Pacinian corpuscles, and Merkel cells was found to be severely decreased in the non-painful presentation of the disease, and almost disappeared in the painful presentation. Furthermore, there was a marked reduction in the expression of axonal and Schwann-like cell markers (with are characteristics of corpuscular denervation) as well as of all investigated mechanoproteins in the non-painful distal diabetic sensorimotor polyneuropathy, and these were absent in the painful form. Taken together, these alterations might explain, at least partly, the impairment of mechanosensitivity system associated with distal diabetic sensorimotor polyneuropathy. Furthermore, our results support that an increasing severity of DDSP may increase the risk of developing painful neuropathic symptoms. However, why the absence of cutaneous mechanoreceptors is associated with pain remains to be elucidated.

Keywords: distal diabetic sensorimotor polyneuropathy; painful and non-painful distal diabetic sensorimotor polyneuropathy; cutaneous sensory corpuscles; human glabrous skin; mechanoproteins

1. Introduction

Diabetic neuropathy includes a group of neuropathies associated with diabetes mellitus, which are the main cause of morbidity and mortality in these patients. The most common complication during the evolution of type 2 diabetes mellitus is distal diabetic sensorimotor polyneuropathy (DDSP) which may affect up to 50% of patients [1], leading

to neuropathic pain in as many as 50% of patients [2,3]. The Toronto Consensus (2011) [2] defined DDSP pain as "*pain that is a direct consequence of abnormalities in the peripheral somatosensory nervous system in diabetic individuals*". Diagnostic of DSSN is frequently delayed due to the scarcity of early diagnostic tests, and recently, axonal swellings in cutaneous biopsies have been proposed as an early marker of sensory nerve injury in type 2 diabetes mellitus [4,5]. Consequently, there is a high risk of ulceration with subsequent distal amputation in lower limbs [6,7]. During the last decade, diagnostic tests such as skin biopsy and corneal confocal microscopy have confirmed its usefulness in the diagnosis of peripheral neuropathies through quantitative analysis of Aδ and C fine intraepithelial nerve fibers [3,8–11]. Thus, more severe small fiber damage in the skin of patients with painful diabetic neuropathy compared with painless diabetic neuropathy has been observed, and the density of intraepithelial nerve fibers was lower in subjects with painful compared with painless neuropathy [11,12].

In addition to pain, light touch and low-frequency vibration are also impaired in DDSP [13–15], suggesting involvement of Aβ fibers and sensory corpuscles (Meissner and Pacinian corpuscles, Merkel cell–neurite complexes; see [16]). Thus, the analysis of sensory corpuscles in cutaneous biopsies has been proposed as a "gold standard" method of diagnostic interest in some peripheral neuropathies and neurodegenerative diseases (see for a review [17,18]).

Focusing on diabetic neuropathy, the seminal article of Ras and Nava [19] in diabetic mice demonstrate a decrease in the number in Meissner-like corpuscles as well as axonal changes. Some years later, Paré et al. [20] observed two phases in corpuscular deterioration in diabetic monkeys. During the first phase, they found a hypertrophy of Meissner corpuscles and Merkel endings, followed by a second phase in which the number of corpuscles declined (but remained higher than in age-matched nondiabetic animals) and the Merkel innervation was reduced (to age-matched nondiabetic levels). Furthermore, the diabetic Meissner corpuscles had an abnormal structure and immunochemistry and Pacinian corpuscles also deteriorated. Data from human skin biopsies revealed a reduction in both Meissner corpuscles and their afferent Aβ myelinated nerve fibers which correlated with decreased amplitudes of sensory/motor responses [21]. Additionally, using in vivo reflectance confocal microscopy, Meissner corpuscles were found reduced in density in diabetes relative to controls [22]. Nevertheless, to the best of our knowledge, a detailed study of human cutaneous sensory corpuscles in painful and non-painful DDSN has not been performed.

Thus, the present study was designed to analyze cutaneous sensory corpuscles from the feet of subjects undergoing painful and non-painful DDSN. We investigated changes in the density, size axonal, and Schwann-like cells of Meissner and Pacinian corpuscles as well as Merkel cell–neurite complexes. Furthermore, we analyzed possible changes in putative mechanoproteins detected on sensory corpuscles [23] which could be at the basis of mechanosensory impairment in DDSN.

2. Materials and Methods

2.1. Patients

Subjects of both genders, free of neurologic disease, who suffered accidental toe amputation (n = 10) were used as the control group. Patients clinically and analytically diagnosed with DM with non-painful (n = 10) or with painful diabetic neuropathy (n = 10) who were subjected to toe amputation due to ischemic complications of DM were also studied. The control skin samples were collected within 6 h after incidental toe amputation at the Service of Plastic Surgery of the Hospital Universitario Central de Asturias, Oviedo, Principality of Asturias, Spain. The skin samples from diabetic patients were collected within 3 h after amputation and were obtained at the Service of Vascular Surgery, Fundación Hopital Jove of Gijón, Principality of Asturias, Spain. The age range of the subjects was 48 to 84 years. The study was approved by the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. CElm, PAst: Proyecto 266/18). All materials were

obtained in compliance with Spanish law (RD 1301/2006; Ley 14/2007; DR 1716/2011; Orden ECC 1414/2013), and according to the guidelines of the Helsinki Declaration II.

Data managing of the diabetic subjects included in the study were divided into 5 parts as follows:

1. Clinical history: age, gender, time of evolution of disease, variant of peripheral neuropathy, HbA1c value, presence of proinflammatory and inflammatory factors (c-reactive protein, erythrocyte sedimentation rate), alterations in blood clotting test, ankle-brachial index, Doppler, and nervous conduction studies.
2. Physical examination: maintained local sensibility, popliteal artery pulse assessment, skin alterations or deformities and allodynia/hyperalgesia/paresthesia/anesthesia. Sensitivity was focused on the clinical examination if anatomical structures and biochemical channels in study are responsible of this sensation.
3. Monofilament testing: to ascertain the presence of sensibility in 4 random points at the affected extremity.
4. Plantar discrimination: capacity, which is closely associated with mechanosensory receptors. Alterations in this variable may be related to an increase, decrease, or absence of mechanoreceptor.
5. DN4 test to estimate neuropathic pain: Previous studies describe how alterations on these sensory structures may produce extreme effects in the form of a total anesthesia in the studied region, or even an excessive painful response under normally painless stimuli. Data from patients and analytical are summarized in Table 1.

Table 1. Date from patients and analytical.

	Control	NP DDSP	P DDSP
Age	62 ± 8.2 s.d.	60 ± 10.3	70 ± 12.4
Gender	mixed	mixed	mixed
Evolution (years)		11 ± 9.4	21 ± 6.5
HBA1C	n.r. (<5.7%)	6.5 ± 0.5	7.6 ± 5.17
C-reactive protein	n.r. (<10 mg/L)	16 ± 18.75	39.5 ± 25.14
GSS	n.r. (0–29 mm/h)	40.5 ± 58.68	129 ± 76.37
Prothrombin rate (%)	n.r. (70–100%)	74.8 ± 40.38	88.6 ± 50.46
Fibrinogen	n.r. (200–400 mg/dL)	526.5 ± 376.7	935 ± 198.15
Ankle-brachial index	n.r. (0.9–1.3 mmHg)	0.79 ± 0.1	not valuable
Echo-Doppler	n.r. (permeable)	permeable popliteal art.	distal obstruction
Sensitivity	n.r. (100%)	Yes (100%)	Yes (50%)
Discriminative capacity	n.r. (<1 cm)	5.5 cm ± 2.17	8 cm ± 3.09
Foot pulses	n.r. (100%)	Yes (33%)	Yes (25%)
Skin deformity	n.r. (normal skin)	Yes (50%)	Yes (25%)
Pain test	not pain	not pain	electric shock
Monofilament test	n.r. (100%)	Positive (50%)	Positive (100%)

NP DDSP: not painful distal diabetic sensorimotor polyneuropathy; P DDSP: painful distal diabetic sensorimotor polyneuropathy; GSS: glomerular sedimentation speed; HBA1C: glycosylated hemoglobin A1C; n.r.: normal range.

The patients included in the study were under different treatments (active principles are in brackets). Those suffering from non-painful DDSP received Bemiparin, Metformin, Efficib (metformin + sitagliptin), Repaglinid, Neparvis (Valsartan + sacubitril), Glargina

Insulin sc; those undergoing painfull DDSP received Velmetia (metformin + sitagliptin), Humalog 200 UI/mL (lispro insulin), Toujeo 300 UI/mL (glargine insulin), Diamicron (gliclazide), Dianben (metformin), Trulicity (dulaglutide). Additonal treatment in non-painful DDSP were anticoagulants and antiagregants: Adiro (acetilsalicilic acide), Sugiran (prostaglandin E1), Trinomía (atorvastatin, acetilsalicilic acide and ramipril), Emcorcor (bisoprolol), Digoxina, Atorvastatin; antihypertensive: Irbersartan, Estatin, Furosemida, Enalapril); and anxiolytics: Lorazepam. Additional treatment in P DDSP were anticoagulants and antiagregants: Pradaxa (dabigatran etexilate), Adiro (acetilsalicilic acide), Atorvastatin, antihy-pertensive (Atenolol, Enalapril, Bisoprolol (bisoprolol fumarato); anxiolytic: Ansium (sulpiride + diazepam), Lorazepan; when analgesic drugs were required also, Paracetamol and Metamizol (magnesium metamizole) were administered.

2.2. Material and Treatment of the Tissues

Skin samples ($n = 30$), 0.5 × 1 × 0.c cm approximately perpendicular to the skin surface were obtained from the plantar aspect of the distal phalanx of toes. The specimens were fixed in 4% formaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 24 h, dehydrated and routinely processed for paraffin embedding.

2.3. Histology and Immunohistochemistry

Hematoxylin-Eosin—Deparaffinized and rehydrated sections were introduced 10 min into Harris Hematoxylin. Subsequently, they were washed in water and passed for 5 s by acidic water (with glacial acetic acid), and finally in an eosin solution for 30 s. The sections were washed, dehydrated, diaphanized in Xylol and mounted with Entellan®.

Single immunohistochemistry—Deparaffinized and rehydrated sections were processed for indirect detection of antibodies (see Table 2), using the EnVision antibody complex detection kit (Dako, Copenhagen, Denmark), following supplier's instructions. Briefly, the endogenous peroxidase activity was inhibited (3% H_2O_2 for 15 min) and the non-specific binding was blocked (10% bovine serum albumin for 20 min). Sections were then incubated overnight at 4 °C with the primary antibody. Subsequently, sections were incubated with the anti-rabbit and anti-mouse EnVision system-labelled polymer (Dako-Cytomation, Santa Clara, USA) for 30 min, washed in buffer solution, and treated with peroxidase blocking buffer (Dako Cytomation). Finally, the slides were washed with buffer solution and the immunoreaction was visualized with diaminobenzidine as a chromogen, then washed, dehydrated, and mounted with Entellan® (Merk, Darmstadt, Germany). To ascertain structural details, the sections were counterstained with Mayer's hematoxylin.

Double immunofluorescence—Sections were also processed for simultaneous detection of PIEZO2, ASIC2 and TRPV4 together with specific markers for Schwann-like cells (S100 protein), axons (Cobo et al., 2021), and for Merkel cells [24,25] (see Table 1). Non-specific binding was reduced by incubating the sections for 30 min with a solution of 25% calf bovine serum in tris buffer solution (TBS). The sections were incubated overnight at 4 °C in a humid chamber with a 1:1 v/v mixture of the polyclonal antibody against PIEZO2, ASIC2 and TRPV4 with monoclonal antibodies against S100 protein, neurofilament protein (NFP), neuron-specific enolase (NSE), cytokeratin 20 (CK20) and chromogranin A (ChrA). After rinsing with TBS, the sections were incubated for 1 h with CFL488-conjugated bovine anti-rabbit IgG (sc-362260, Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1:200 in TBS, them rinsed again and incubated for another hour with CyTM3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch, Baltimore, MD, USA) diluted 1:100 in TBS. Both steps were performed at room temperature in a dark humid chamber. Sections were finally washed, and the cell nuclei were stained with DAPI (10 ng/mL). Triple fluorescence was detected using a Leica DMR-XA automatic fluorescence microscope (Microscopía fotónica y Proceso de imágen, Servicios científico-técnicos, Universidad de Oviedo) coupled with a Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany) and the images captured were processed using the soft-

ware Image J version 1.43 g Master Biophotonics Facility, Mac Master University Ontario (www.macbiophotonics.ca (access on 11 January 2021)).

Table 2. Primary antibodies used in the study.

Antigen (Clone)	Origin	Dilution	Supplier
Axonal markers			
NSE (BBS/NC/IV-H14)	Mouse	1:100	Dako, Glostrup, Denmark
NFP (NF-H-RNF402)	Mouse	1:200	Santa Cruz Biotechnology, CA, USA
Schwann-related cells			
S100P	Rabbit	1:5000	Dako, Glostrup, Denmark
S100P (4C4.9)	Mouse	1:1000	Thermo Scientific, Freemont, CA, USA
Merkel cells			
ChrA (DAK-A3)	Mouse	Prediluted	Dako, Glostrup, Denmark
CK20 (ks 20.8-IS777)	Mouse	Prediluted	Dako, Glostrup, Denmark
Ion channels			
ASIC 2	Rabbit	1:200	Lifespan Biosciences, Seatle, WA, USA
TRPV4	Rabbit	1:200	Abcamn, Cambridge, UK
PIEZO2	Rabbit	1:500	Sigma-Aldrich, Madrid, Spain

ASIC2: acid-sensing ion channel 2; ChrA: chromogranin A; CK20: cytokeratin20; NFP: neurofilament proteins; NSE: neuron-specific enolase; S100P: S100β protein; TRPV4: transient receptor potential vanilloid 4.

For control purposes, representative sections were processed in the same way as described, using non-immune rabbit or mouse sera instead of the primary antibodies or omitting the primary antibodies in the incubation. Furthermore, when available, additional controls were carried out using specifically preabsorbed antisera. Under these conditions, no positive immunostaining was observed (data not shown).

2.4. Quantitative Study

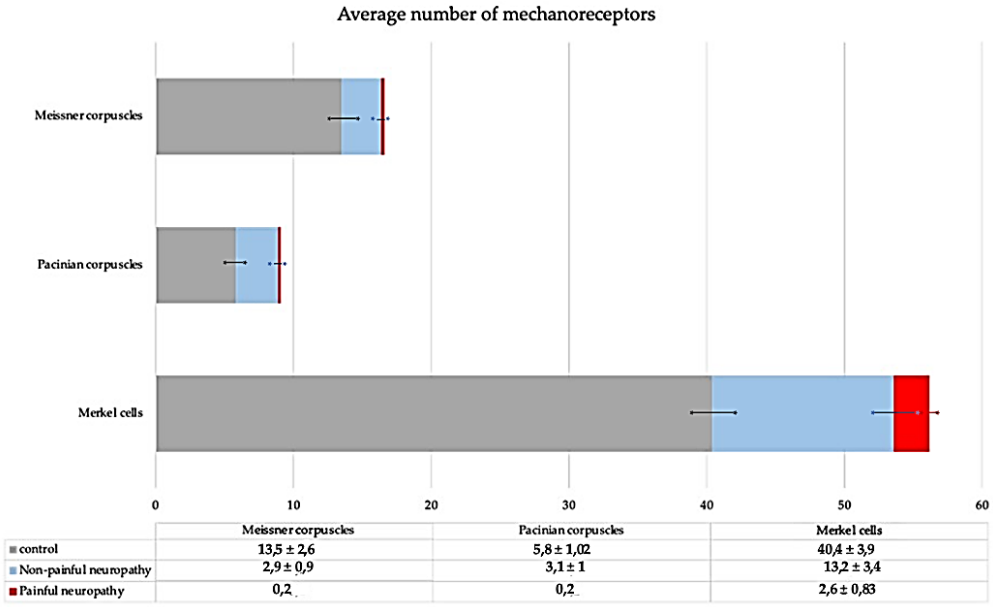
Quantitative analyses were performed to determine the density of cutaneous digital sensory corpuscles. We examined 500 sections of glabrous toe skin from controls (n = 100), NP DDSP (n = 200) and P DDSP (n = 200) subjects, to evaluate the density of sensory corpuscles. The number of Merkel cells, as well as Meissner and Pacini corpuscles was calculated as follows: 20 fields were quantified in microscopy at 10×, per individual, in 5 sections separated by 50 µm, by two different observers, and the results obtained were averaged. Data are expressed as mean ± SD/mm^2. In turn, to verify the functionality of the nervous structures under study, the percentage of positive PIEZO2 mechanoreceptors was estimated, performing the quantification with double immunofluorescence as described below: the percentage of presumably functional Merkel cells was quantified by the simultaneous detection of PIEZO2 and CK20 (as specific marker) in 5 sections separated from each other by 50 µm, while for the percentage of positive Meissner and Pacinian PIEZO2 corpuscles, the PIEZO2 and S100P antibodies (as specific marker for Schwann cells) were used, using the following formula: % PIEZO2+: (Average number of total PIEZO2 + mechanoreceptors/Average number of total mechanoreceptors) × 100.

3. Results

3.1. Quantitative Analyses of Cutaneous Sensory Corpuscles: Association between Density and Neuropathy

A total of 168 Meissner corpuscles were analyzed, all of them identified since the expression of S100P by the Schwann-like (lamellar) cells. Meissner corpuscle density was lower in neuropathy patients compared with controls: there was a reduction of about 80%

in NP DDSP, and an almost complete absence in P DDSP (only 2 Meissner corpuscles were identified in this group of patients) (Figure 1).



	Meissner corpuscles	Pacinian corpuscles	Merkel cells
control	13,5 ± 2,6	5,8 ± 1,02	40,4 ± 3,9
Non-painful neuropathy	2,9 ± 0,9	3,1 ± 1	13,2 ± 3,4
Painful neuropathy	0,2	0,2	2,6 ± 0,83

Figure 1. Average number of Meissner corpuscles, Pacinian corpuscles, and Merkel cells in the glabrous skin of the toes in control (grey), non-painful DPN (blue), and painful DPN (red) for 20 fields at 10×. Data are expressed as mean ± SD/mm^2. A reduction in the number of all types of sensory corpuscles studied was detected with disease, in fact, the most dramatic decrease was in painful-DPN subjects. * is S.D.

The results obtained in Pacinian corpuscles (identified because of morphology and the occurrence of S100P in the Schwann-like cells; n = 58) were parallel to those of Meissner corpuscles: there was a reduction of about 50% in NP DDSP, and an almost complete absence in P DDSP (Figure 1).

Regarding Merkel cells (identified by the expression of CK20), significant differences were also found between the controls and DDSP. In the NP DDSP, there was a reduction of about 68% with respect to the controls, and in P DDSP the reduction reached 93%. However, 7 patients from this group maintained a density of Merkel cells similar to the controls (Figure 1).

3.2. Immunohistochemical Profile of Meissner and Pacinian Corpuscles

Meissner corpuscles were studied for detection of the axonal markers NFP and NSE, and Schwann-like cells for detection of S100P (see [16]). Meissner corpuscles were identified at all three groups analyzed, although in P DDSP they were found in only 2/10 subjects.

Differences in the morphology, size, number, placement within dermis, and intensity of immunostaining were observed among the three groups evaluated. In the control group, Meissner corpuscles were elongated and were always localized in the dermal papillae. The lamellar cells were packed, arranged in parallel, and displayed strong S100 protein immunoreactivity (Figure 2a,b). The axons showed immunoreactivity for NFP which run all over the corpuscle (Figure 2c), in an irregular course among lamellar cells (Figure 2d). In the NP DDSP patients, the predominant localization of Meissner corpuscles also was within the dermal papillae, but some were also displaced to the dermis (Figure 2e). Furthermore, the size was reduced, and the morphology was rounded. The lamellar cells were disorganized and unstructured form (Figure 2e,f), and the axon was sometimes unidentified (Figure 2g,h). The scenario changed dramatically in the P DDSP group in which Meissner corpuscles were not identified, and when identified morphologically they

lacked S100P and NF protein (Figure 2i–k). Interestingly, in the dermis of P DDSP, abundant S100P-positive dendritic cells were observed (Figure 2i,j).

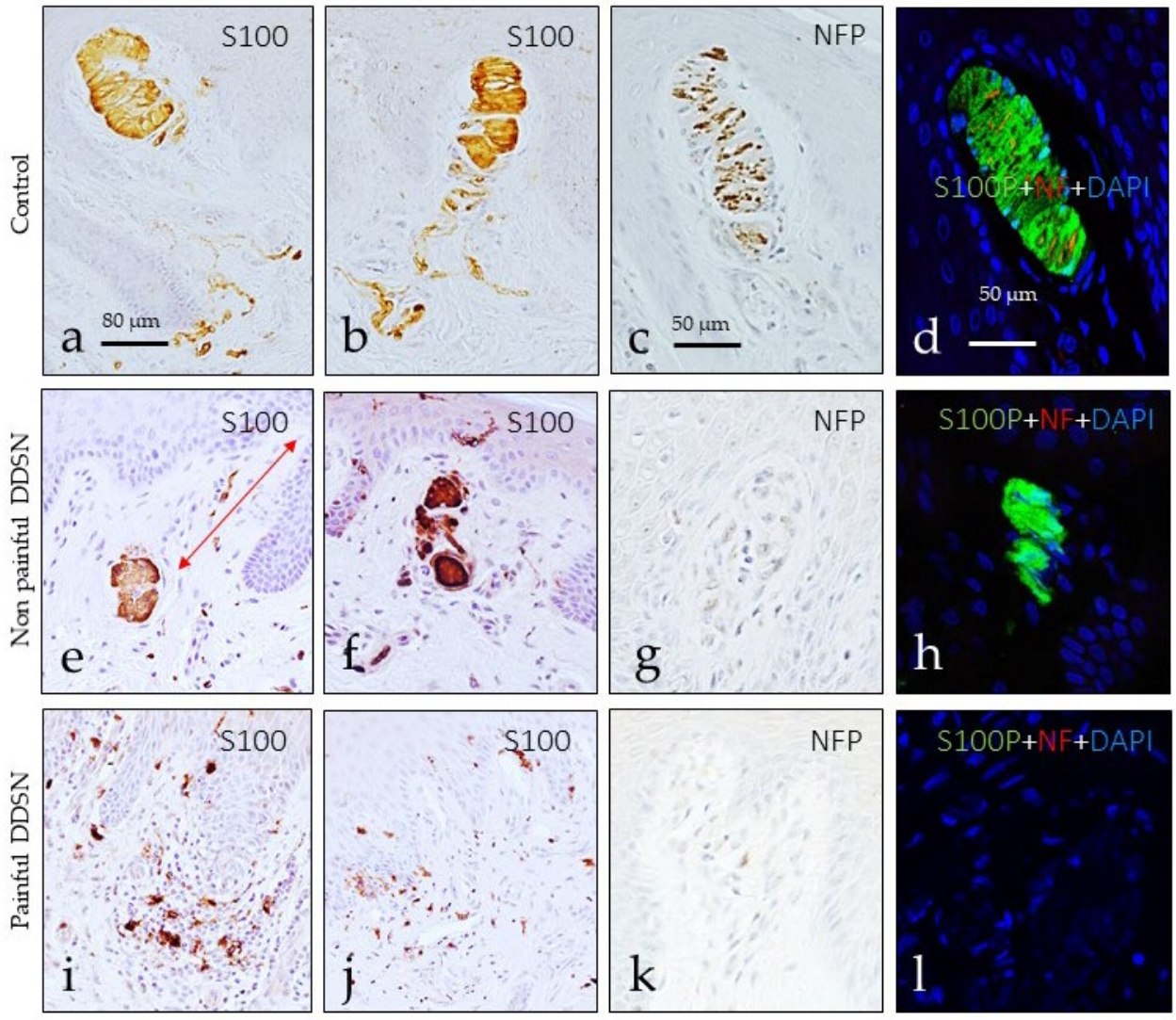


Figure 2. Meissner corpuscles from the glabrous toe skin of subjects belonging to each group studied. S100 protein (S100P) was used to immunolabel the lamellar cells, and neurofilament protein to immunolabel the axons. In controls, Meissner corpuscles were located inside the dermal papillae, and both the lamellar cells and the axons displayed a typical aspect (**a**–**d**). The Meissner corpuscles from NP DDSP subjects were mainly placed in the dermis, outside the dermal papillae, were smaller and the lamellar cells disarranged while no-positivity for NFP was detected (**e**–**h**). In P DDSP Meissner corpuscles were lost (**i**–**l**) and large infiltrated of dendritic S100P-positive cells were observed. S100: S100 protein; NFP: neurofilament protein; NF: neurofilament; DAPI: 4′,6-diamidino-2-phenylindole.

Mechanotransduction is the process which converts mechanical forces in action potentials, and in the skin, it occurs in mechanoreceptors, including Meissner and Pacinian corpuscles, and Merkel cell–neurite complexes (see [23]). In this process, some mechanogated ion channels such as ASIC2, TRPV4, and, primarily, PIEZO2 [23] participate. Thus, we have investigated the possible changes in these mechanoproteins within sensory corpuscles during DDSP as a possible partial explanation of touch changes found in this disease. Using double immunofluorescence associated with confocal microscopy, we observed the occurrence of PIEZO2 (Figure 3a), ASIC2 (Figure 3d), and TRPV4 (Figure 3g) restricted to the axon, but not in the lamellar cells of Meissner corpuscles of control subjects. Immunofluorescence for all three mechanoproteins investigated was absent from Meissner corpuscles of both NP DDSP (Figure 3b,e,h) and P DDSP (Figure 3c,f,i).

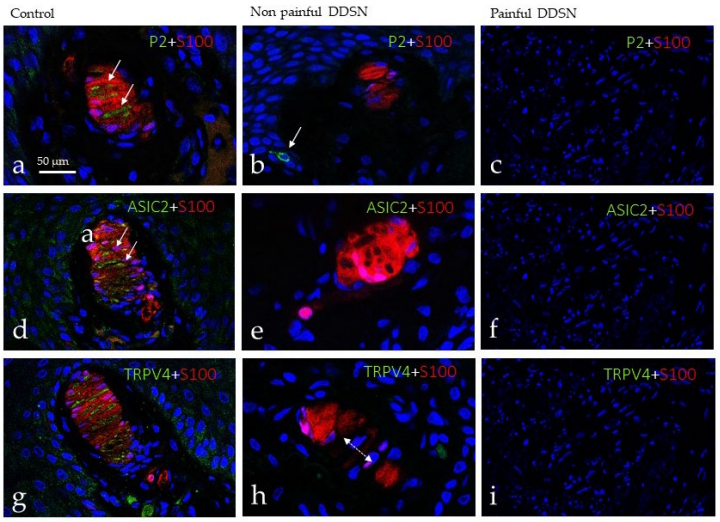


Figure 3. Double immunofluorescence for S100 protein (red fluorescence) and PIEZO2, ASIC2 and TRPV4 (green fluorescence) in Meissner corpuscles of glabrous toe skin of control (**a**,**d**,**g**), NP DDSP (**b**,**e**,**h**), and P DDSP (**c**,**f**,**i**) patients. Sections were counterstained with DAPI to ascertain structural details. In controls PIEZO2, ASIC2 and TRPV4 were regularly detected in the axons, were all these mechanoproteins were undetectable in the corpuscles of NP DDSP and P DDSP patients. P2: Piezo 2; ASIC2: Acid sensing-ion channel; TRPV4: Trasient receptor potential cation channel V4.

Evident changes were also noted in Pacinian corpuscles of DDSP patients with respect to the controls (Figure 4). The Pacinian corpuscles from normal subjects showed the typical onion-layer arrangement. The axon was placed at the center (displaying NFP immunoreactivity) of the corpuscle and was encircled by the lamellae of the Schwann-like cells that form the inner core (positive for S100 protein); outside these neural components are the intermediate layer, the outer core, and the capsule derived from the endoneurium and the perineurium (Figure 4a–c). In subjects with NP DDSP, the neural compartment of the Pacinian corpuscles (i.e., the axon and the inner core) was disarranged (Figure 4d,e), and no immunoreactivity for NFP was detected, nor in most axons (it was present in about 10%) or S100 protein (it was detected in about 15%). Nevertheless, the Pacinian corpuscles can be identified based on their morphology. Finally, in the Pacinian corpuscles from P DDSP patients, no immunoreactivity was detected for NFP or S100 protein (Figure 4g–i).

Regarding the investigated mechanoproteins PIEZO2, ASIC2, and TRPV4 they were detected restricted to the axon of Pacinian corpuscles from control subjects. Conversely, they were absent from the axon of both NP DDSP and P DDSP (Figure 5).

3.3. Immunohistochemical Profile of Merkel Cells

Merkel cells are epidermal cells localized in the basal stratum of the epidermis that is selectively immunolabelled with antibodies against cytokeratin 20 (CK20) or chromogranin A (ChrA). Using the expression of these proteins as a marker, Merkel cells were found in the epidermal rete pegs of all subjects investigated but the number was reduced in DDSP patients, and the number of cells decrease with neuropathic (Figures 6 and 7). In the skin of control subject clusters of up to four Merkel cells were regularly observed (Figure 6a,d,g,j), whereas in NP DDSP (Figure 6b,e,h,k) and P DDSP patients (Figure 6c,f,i,l) Merkel cells were found scattered in the basal stratum of nerve-formed clusters. Nevertheless, the remaining cells retained the basic immunohistochemical profile for CK20 or ChrA.

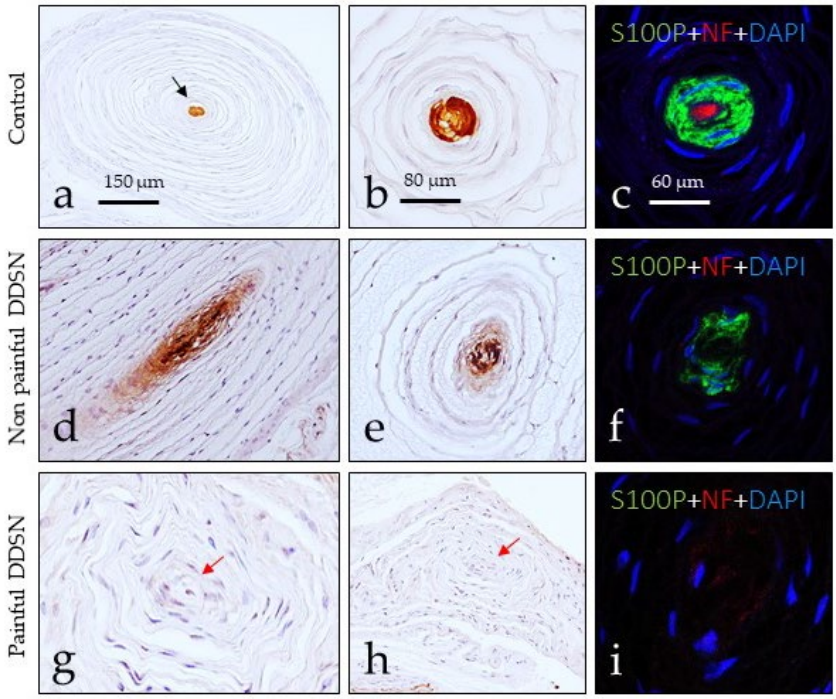


Figure 4. Immunohistochemical localization of S100 protein (S100P) in the inner core cells and neurofilament protein (NFP) in axon of human toes Pacinian corpuscles from control (**a**–**c**), NP DDSP (**d**–**f**) and P DDSP (**g**–**i**) patients. The axon was identifiable only in control group (red fluorescence **c**,**f**,**i**) while S100 protein (S100P) immunolabeling was found in the inner core of control and NP DDSP patients whereas it was undetectable in P DDSP (red arrows in (**g**,**h**)).

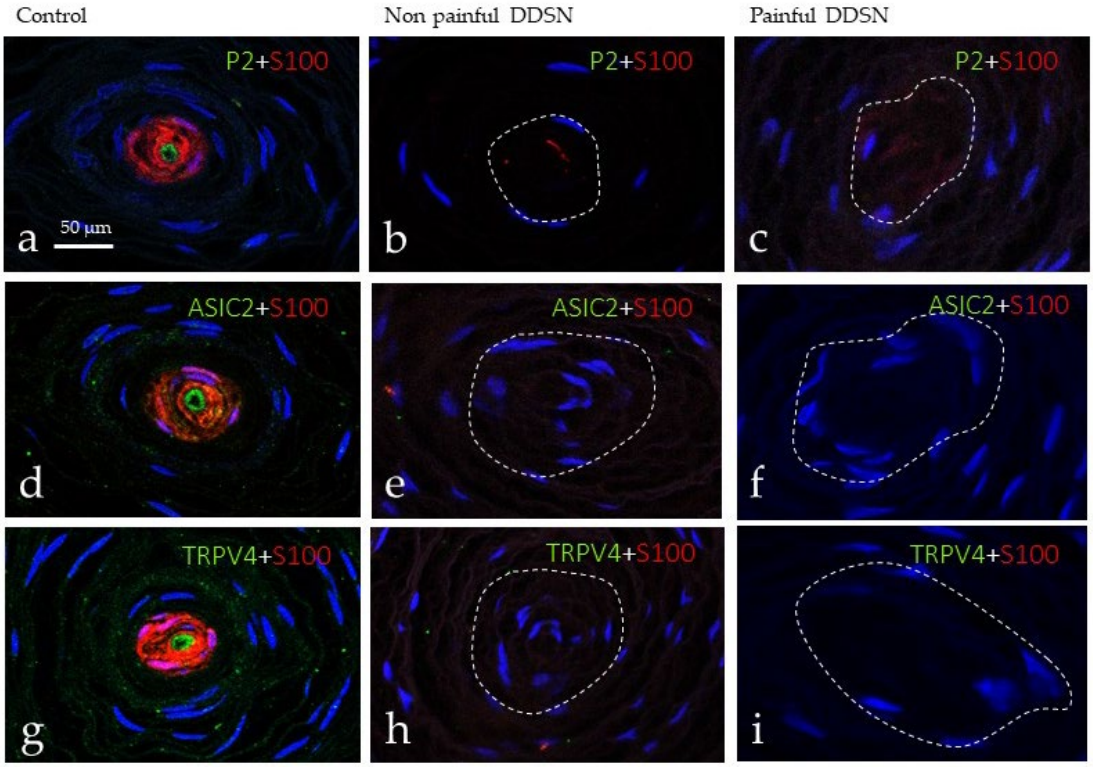


Figure 5. Double immunofluorescence for S100 protein, PIEZO2 (P2; (**a**–**c**)), ASIC2 (**d**–**f**) and TRPV4 (**g**–**i**) in cutaneous Pacinian corpuscles from control (**a**,**d**,**g**), NP DDSP (**b**,**e**,**h**), and P DDSP (**c**,**f**,**i**) patients. PIEZO2, ASIC2, and TRPV 4 were presented only in the axon of Pacinian corpuscles.

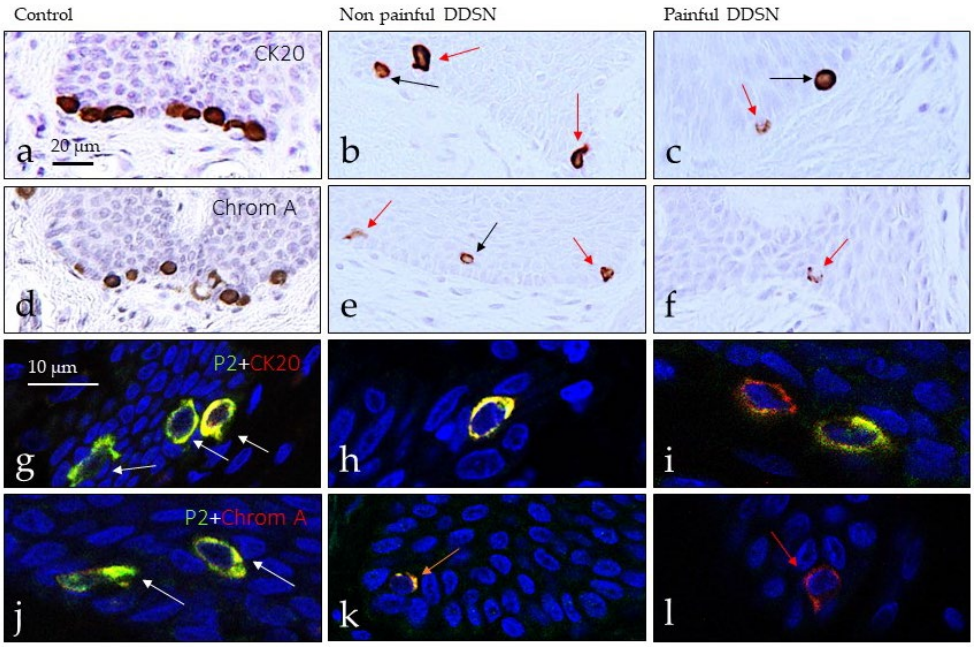


Figure 6. Cutaneous Merkel cells were identified since the localization within the basal stratum of the epidermis, and the expression of cytokeratin (CK20) and chromogranin A (ChrA). They were identified in control subjects (**a**,**d**,**g**,**j**) and DDSP patients (**b**,**e**,**h**,**k**); (**c**,**f**,**i**,**l**). In control, Merkel cells formed clusters whereas in diabetic patients were scattered and reduced in number. Merkel cells displayed PIEZO2 in both normal and pathological conditions (**g**–**l**). Ck20: Cytokeratin 20; Chrom A: Chromogranin A.

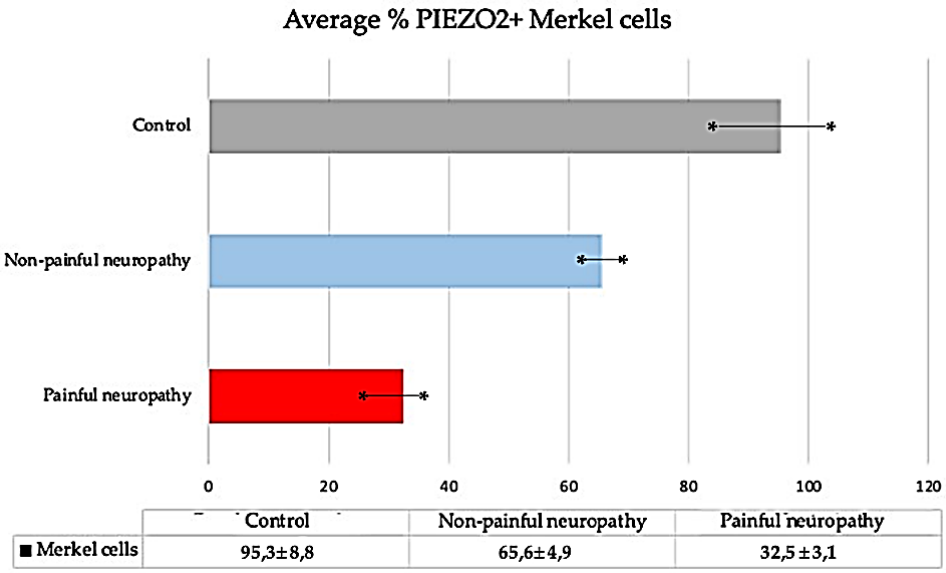


Figure 7. Average (expressed as mean ± DS) of PIEZO2-positive Merkel cells in the skin of control, NP DDSP and P DDSP subjects. A significant reduction in the number of Merkel's cells was observed with disease. * is S.D.

To investigate whether Merkel cells were mechanosensitive, we analyzed the occurrence of PIEZO2 within them [26]. PIEZO2 was observed co-localized with CK20 (Figure 6g–i)- and ChrA (Figure 6j–l)-positive cells, thus were identified as Merkel cells. However, the density of Merkel cells and PIEZO2-positive Merkel cells progressively decreased with the evolution of disease (Figure 7).

4. Discussion

The present study was designed to investigate the changes in cutaneous sensory corpuscles and Merkel cell during diabetic neuropathic. This topic has been partially analyzed although it is broadly accepted that diabetic neuropathy is accompanied by a progressive impairment in the somatosensory system that affects the quality of life of patients [27,28]. This deterioration involves all levels of the somatosensory pathways from the skin with the cutaneous peripheral somatosensory receptors (free nerve endings, sensory corpuscles, Merkel cell–axonal complexes); see [16,29] for the cerebral cortex [28,30,31]. In the study we used skin to samples from control subjects as well as from patients suffering NP DDSP and P DDSP to analyze the cutaneous Meissner and Pacinian corpuscles, as well as Merkel cells–axonal complexes. We used immunohistochemistry associated with a battery of antibodies to identify the axon and Schwann related cells (since both are involved in diabetic neuropathy [32,33]) and a series of putative mechanoproteins (PIEZO2, ASIC2 and TRPV4) which are involved in mechanotransduction [23].

The effects of diabetic neuropathy on sensory loss and pain have been reviewed in detail by Shillo et al. [30] and Rosenberger et al. [31], and although most studies support a correlation between neuropathy severity and neuropathic pain in DDSP [34,35], others do not [36]. In any case, the evidence suggests that an increasing severity of DDSP may increase the risk of developing painful neuropathic symptoms thus frequently evolving to P DDSP [2,37].

It is now accepted that cutaneous sensory corpuscles share the cell composition and immunohistochemical profile of the sensory fibers of which they depend. Therefore, their evaluation through cutaneous biopsy can be a useful method to evaluate and follow-up DDSP evolution and/or treatment. Early studies have reported a decrease in the density and structural deterioration of sensory corpuscles in diabetic monkeys [20] (Paré et al., 2007) and humans [21]. As far as we know, a detailed study in humans differentiating between NP DDSP and P DDSP was never carried out.

On the other hand, studies on human sensory corpuscles in DDSP are limited and do not use specific immunohistochemistry assays for the neural component (i.e., axon and Schwann-related cells). In the present study, we observed that progression from NP DDSP to P DDSP courses with a reduction in size of Meissner corpuscles, changes in the morphology and cellular arrangement, displacement to deep dermis, and loss of immunoreactivity for axonal and Schwann-like cell markers (Figure 8). The reduction or absence of S100 protein in lamellar cells, together with absence of NFP immunostaining, strongly suggest denervation of those corpuscles [38–40].

Our results on NP DDSP are in good agreement with those reported by Peltier et al. [31] in the digital glabrous skin. These authors observed that in diabetes type II, Meissner corpuscles decrease and become disorganized, and these changes are attributed to axonal loss. However, to our knowledge, the absence of Meissner corpuscles that occurs in P DDSP has not been previously reported. Thus, it seems that the transition from non-painful to painful DDSP is due to a loss of Meissner corpuscles and therefore a loss in tactile discrimination.

Regarding Pacinian corpuscles, the effects of DDSP were evident in both number and structure and are consistent with the data reported by Pare et al. [30] in Pacinian corpuscles of diabetic monkey. These authors observed that Pacinian corpuscles showed a pronounced disruption consisting of breakdown in the inner lamella, irregular spacing between lamellae, and thickening of the outer lamellae. In the present study, we have found a marked decrease in the number of Pacinian corpuscles associated with diabetes neuropathic, in fact a dramatic reduction was observed in P DDSP. It must be noted that Pacinian corpuscles of NP DDSP patients showed inner core destruction whereas painful-DPN patients both the inner core and the complex outer core/capsule are affected by disease (Figure 8). As far as we know, these changes have not been reported earlier.

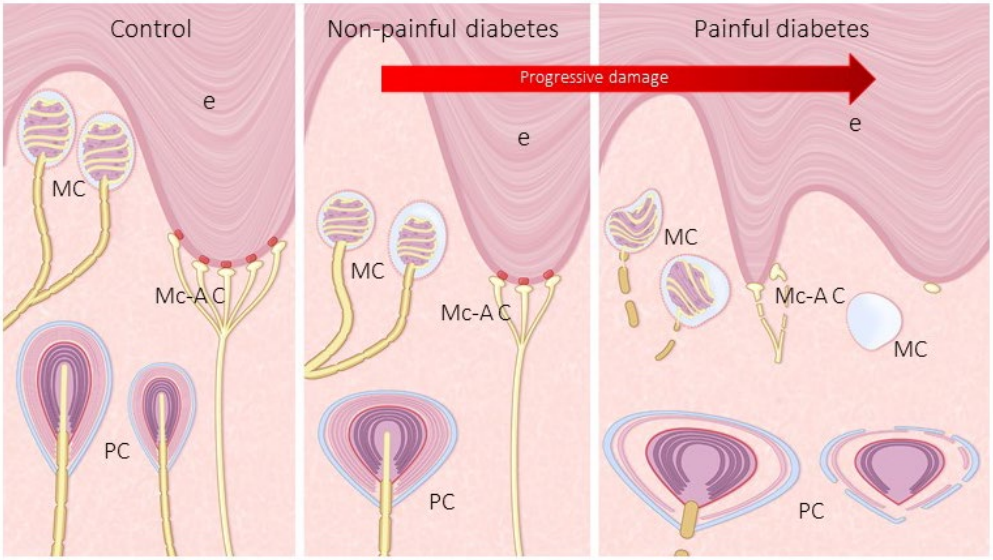


Figure 8. Schematic representation of cutaneous sensory corpuscles (MC: Meissner corpuscles; PC: Pacinian corpuscles) and Merkel cell–axonal complexes (Mc-Ac) in the skin of human toes from control NP DDSP and P DDSP subjects. DDSP results in a reduction in the density of all three types of mechanoreceptors, changes in their placement and morphology, as well as in their immunohistochemical profile and expression of putative mechanoproteins. E:epidermis; PC: pacinian corpuscles.

In relation with Merkel cells our results disagree with those from Pare et al. [30]. These authors reported an increase in Merckel cells in the shorter-term hyperglycemic monkeys, whereas we have found an important reduction in number of Merkel's cells associated with DDSP, especially P DDSP. Furthermore, this condition results in absence of the typical clusters of Merkel cells in normal skin.

During the last decade it has been definitively established that the different qualities of somatosensitivity depend on the expression of different ion channels in specific subtypes of primary sensory neurons and their peripheral terminals. In particular, PIEZO2, ASIC2, TRPV4, and TRPC6, participate in different modalities of mechanosensitivity (see for a review [23]). Previous studies from our laboratory have demonstrate the occurrence of PIEZO2, ASIC2, TRPC6 and TRPV4 in the axons, of ASIC2 and TRPV4 in the corpuscular Schwann-related cells, and of PIEZO2 and ASIC2 in the Merkel cell–neurite complexes. Our results in the skin of control subjects are in complete agreement with previous data. Interestingly, all those proteins disappeared from the axon of both groups of DDSP, thus lending further support to the observation of absence of NFP-positive axons, and to the tactile impairment of touch in diabetic patients (Figure 8). Nevertheless, further studies are necessary to confirm the correlation between mechanoproteins depletion in cutaneous mechanoreceptors and tactile and vibration alterations in diabetes [13,30,31].

5. Conclusions

Overall, present results demonstrate that Meissner and Pacinian corpuscles and Merkel cells (a part of the Merkel cell–axonal complex), which represent the most peripheral part of the mechanosensory system, undergo progressive topographical, morphological, and structural changes from non-painful to painful DDSP. These changes also affect the expression of axonal and Schwann-like cell proteins, as well as some putative mechanoproteins. These variations are in good agreement with previous experimental studied but in those studies a differential between non-painful and painful DDSP cannot be established. Furthermore, corpuscular alterations observed here can explain the tactile defects associated with DDSP, but not the presence of pain. Nevertheless, our results support that an increasing severity of DDSP may increase the risk of developing painful

neuropathic symptoms. Nociception is associated with free nerve endings [29], which were not investigated in this study and have been found reduced in diabetic neuropathy [11,12]. In our opinion the occurrence of pain when mechanoreceptors deteriorate could be related with changes in the circuitry of the dorsal horn of the spine affecting the gate-control of pain (predominance of nociceptive inputs vs. decrease in mechanical inputs) or changes in non-neuronal cells especially microglia [41,42]. Nevertheless, a contribution of local ischemia to deterioration of sensory corpuscles and pain cannot be ruled out.

Author Contributions: Conceptualization, Y.G.-M. and O.G.-S.; methodology, J.G.-P.; software, J.G.-P.; validation, M.G.-G., I.M. and E.V.; formal analysis, O.G.-S.; investigation, Y.G.-M., J.G.-P., J.M.-C., J.F. and O.G.-S.; J.G.-P.; resources, M.G.-G. and I.M.; data curation, Y.G.-M., E.V. and J.M.-C.; writing—original draft preparation, Y.G.-M., J.F. and O.G.-S.; writing—review and editing, T.C. and O.G.-S.; visualization, Y.G.-M. and T.C.; supervision, O.G.-S. and T.C.; project administration, J.F.; funding acquisition, J.F. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by Institutional Review Board of the Fundación Jove Hospital and the Ethical Committee for Biomedical Research of the Principality of Asturias, Spain (Cod. CElm, PAst: Proyecto 266/18).

Informed Consent Statement: Written informed consent was obtained by I.M. and M.G.-G. from all patients prior to any procedure.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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3.8. Resultados – Publicación 8

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Merkel Cell Carcinoma Display PIEZO2 Immunoreactivity

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Abstract: As an essential component of mechano-gated ion channels, critically required for mechanotransduction in mammalian cells, PIEZO2 is known to be characteristically expressed by Merkel cells in human skin. Here, we immunohistochemically investigated the occurrence of Piezo channels in a case series of Merkel cell carcinoma. A panel of antibodies was used to characterize Merkel cells, and to detect PIEZO2 expression. All analyzed tumors displayed PIEZO2 in nearly all cells, showing two patterns of immunostaining: membranous and perinuclear dot-like. PIEZO2 co-localized with cytokeratin 20, chromogranin A, synaptophysin and neurofilament. Moreover, neurofilament immunoreactive structures resembling nerve-Merkel cell contacts were occasionally found. PIEZO2 was also detected in cells of the sweat ducts. The role of PIEZO2 in Merkel cell carcinoma is still unknown, but it could be related with the mechanical regulation of the tumor biology or be a mere vestige of the Merkel cell derivation.

Keywords: merkel cells; merkel cell carcinoma; PIEZO2; mechanobiology; cancer mechanobiology; ion channels

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1. Introduction

Merkel cell carcinoma (MCC) is a rare, clinically aggressive neuroendocrine tumor of the skin with high propensity for local, regional, and distant spread [1–4], being lethal in about 3–35% of cases [5]. MCC fundamentally qoccurs in elderly people, being sun exposure or immunosuppression known risk factors [6]. MCC nature has still some controversy: Sunshine et al. [7] recently hypothesized both Merkel cells (MC) and dermal fibroblasts might be on the cellular origin. Epidermal stem cells, keratinocytes and Pro-B or Pre-B cells have been also proposed as the cellular origin [2]. In any case, most evidences lend support to an origin from MC [8]. The etiology for MCC is currently divided between Merkel cell polyomavirus (MCPyV) and solar exposure with UV-induced mutations [9,10].

MCs are highly specialized epithelial cells located in the epidermal basal layer and also in the external portion of hair follicles. Classically, they were regarded as nonkeratinocyte epidermal "tastzellen" or "touch cell" that functions as a tactile skin receptor [11]. Currently, it has been definitively demonstrated that they are essential components of the Merkel cell-neurite complexes acting together as type I slowly-adapting low-threshold mechanoreceptors by transforming tactile stimuli into $Ca2^{+}$-action potentials [12], inducing release of neurotransmitters and activating Aβ-afferent nerve endings [13,14].

Mechanically gated ion channels are at the origin of these mechanically induced action potentials, in a process called mechanotransduction [15]. Among them, members of PIEZO family are essential components of distinct stretch-activated ion channels capable to perform mechanotransduction thanks to their particular tridimensional arrangement [16], being expressed in a wide range of normal and neoplastic tissues [17–19]. PIEZO proteins represent a new class of mechanosensitive channels, which respond to mechanical forces and allow $Ca2^{+}$ influx in the cell. PIEZO1 and PIEZO2 are the only members of PIEZO family, characterized by a high grade of homology and a similar mechanosensory function [18,19]. They are assumed (especially PIEZO1) to participate in various mechano-associated biologic processes such as sensing of shear stress (particularly in the vasculature), bladder distention and regulation of urine flow, volume regulation, cellular development, proliferation, migration and elongation [19,20].

Although both PIEZO channels share a great homology, PIEZO1 is a more polymodal sensor for mechanical forces, detecting a larger number of stimuli, while PIEZO2 is more narrowly tuned to specifically detect mechanical touch [19]. Thus, PIEZO2 is present in peripheral sensory neurons and cutaneous low-threshold mechanoreceptors [21–23], as well as in MCs [13,22,24,25]. Particularly regarding MCs, both cellular density and PIEZO2 expression diminish with age in glabrous digital skin [26], which makes contrast with the elderly orientation of MCC. Presumably the behavior of MC is different in other anatomical placements. Whether MCC also express PIEZO proteins has been never investigated, although PIEZO2 presence in cultured human MCC-13 cells has been probed [27]. Therefore, the present study was designed to investigate the occurrence of PIEZO2 in MCC. This could be of potential interest because of the potential role of mechanosensitive ion channels in diagnosis, prognosis or treatment of cancer [15,16,28].

2. Materials and Methods

Samples of histologically diagnosed MCC were analyzed to perform the research. The localization of the tumors was predominantly the head (n = 7, corresponding to ear, parietal region, eyelid, nose and cheek), arm (forearm and hand, n = 3) and leg (n = 3). The age range was between 70 and 92 years (8 females and 5 males). All of these materials were obtained in compliance with The Spanish Law (RD 1301/2006; Ley 14/2007; DR 1716/2011; Orden ECC 1414/2013) and the study was approved by the Ethical Committee for Biomedical Research of the Complejo Asistencial Universitario de Salamanca, Spain (Cod. CElm: PI2022 02 935). The specimens were fixed in 4% formaldehyde (0.1 M phosphate-buffered saline; pH 7.4) and embedded in paraffin as usual. The pieces were cut perpendicularly to the skin surface into 10 µm thick sections and mounted on gelatin-coated microscope slides.

Immunohistochemistry was performed using the automated diagnostic platform Leica Bond III with the Leica Bond™ Polymer Refine Detection Kit (Leica Biosystems™, Newcastle upon Tyne, UK), following the manufacturer's instructions.

Primary antibodies used in the study are listed in Table 1. Antibodies against cytokeratin 20 (CK20), synaptophysin (Syn), chromogranin A (ChrA), and neurofilament proteins (NFP) were used to identify and characterize MC. Sections were incubated with primary antibodies for 1 overnight at 4 °C in a dark humid chamber. After rinsing, Dako EnVision System labeled polymer-HR anti-rabbit IgG or anti-mouse IgG (DakoCytomation, Glostrup, Denmark) was applied for 30 min at room temperature. Immunoreaction was visualized by using 3-3′-diaminobenzidine as chromogen. Finally, sections were counterstained with hematoxylin-eosin in order to ascertain structural details. For control purpose, sections were incubated without primary antibodies and/or secondary antibodies, employing non-immune rabbit/mouse sera instead primary antibodies.

On the other hand, double immunofluorescence was performed in order to investigate the co-localization of PIEZO2 with the above MC markers, as well as the possible relationship of MCC cells with neural structures. Skin sections were deparaffinized and rehydrated, and the non-specific binding was reduced by bovine serum albumin (5% in Tris Buffer Saline; pH 7,4) for 30 min. Sections were incubated overnight, at 4 °C, in a dark

humid chamber with a 1:1 v/v mixture of anti-PIEZO2 and anti-CK20; or anti-PIEZO2 and anti-Syn; or anti-PIEZO2 and anti-ChrA; and anti-PIEZO2 and NFP. Once the sections were rinsed, they were incubated with Alexa fluor 488-conjugated goat anti-rabbit IgG (Serotec™, Oxford, UK, diluted 1:1000) and Cy 3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch™, Baltimore, MD, USA, diluted 1:50), one after another, in a dark humid chamber, for 1 h, at room temperature. Finally, nuclei were labelled with DAPI (10 ng/mL). Immunofluorescence was detected by using a Leica DMR-XA automatic fluorescence microscope coupled with a Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Heidelberg, Germany). Additionally, control sections were processed as previously described, but primary antibodies were either omitted in the incubation or substituted by non-immune rabbit/mouse sera.

Table 1. Primary antibodies used in the study.

Antigen	Origin	Dilution	Supplier
Merkel cell markers			
CK20 (clone Ks20.8-, IS777)	Mouse	Prediluted	Dako [1]
Chromogranin A	Mouse	Prediluted	Leica [2]
NFP (clone 2F11)	Mouse	1:100	Dako [1]
Synaptophysin (clone DAK-SYNAP)	Mouse	1:100	Dako [1]
PIEZO2	Rabbit	1:200	Milipore Sigma [3]

CK20: cytokeratin 20; NFP: neurofilament. [1] Glostrup, Denmark; [2] Newcastle upon Tyne, United Kingdom; [3] Burlington, MA, USA.

3. Results

Merkel cells from human skin displayed immunoreactivity for CK20 and ChrA, which well co-localized with PIEZO2 (Figure S1). Structurally MCC formed cords, trabeculae or sheets of small monomorphic cells that occasionally contained blood vessels. The assessment and diagnosis of MCC was performed based on the expression of markers such as CK20 (Figure 1a,b), chromogranin A (Figure 1c,d), synaptophysin (Figure 1e,f) or NFP (Figure 1g,h), although the proportion of NFP positive cells was generally low.

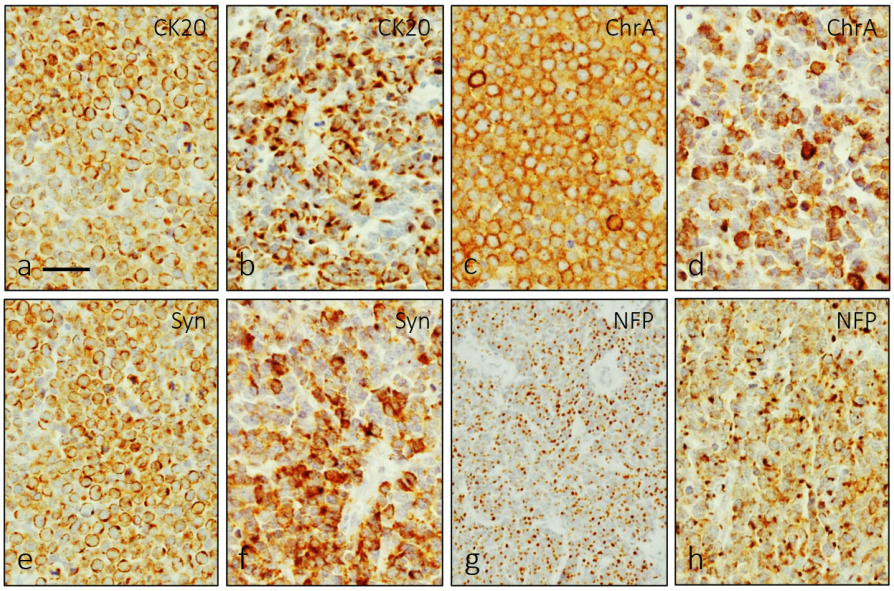

Figure 1. Single immunohistochemistry for CK20 (**a**,**b**), ChrA (**c**,**d**), Syn (**e**,**f**) and NFP (**g**,**h**) was used to characterize MCC cells. They showed two patterns of immunostaining: membranous perinuclear and dot-like. Scale bar 20 μm.

PIEZO2 immunoreactivity was detected in all the cases, with a characteristic cytoplasmic pattern of distribution in the cells expressing CK20 (Figure 2a,b). The general pattern consisted in an overlap between membranous (Figures 2c–f and 3a,b) and paranuclear dot-like (Figures 2g,h and 3c,d) patterns.

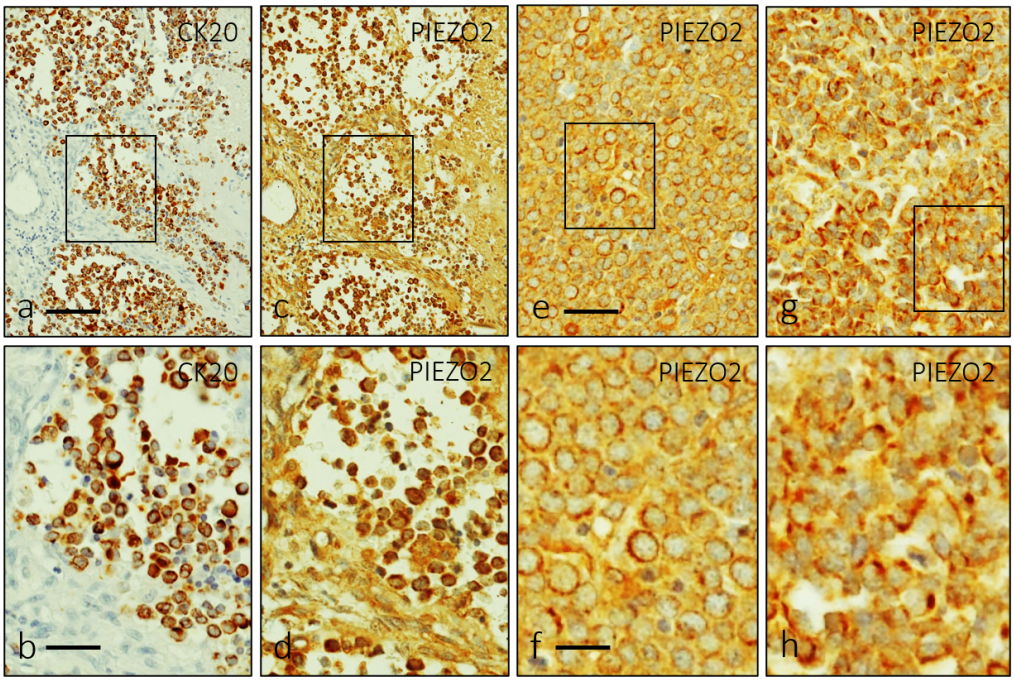

Figure 2. Single immunohistochemistry for CK20 (**a**,**b**) and PIEZO2 (**c**–**h**) in approximate serial sections showing a perinuclear pattern of distribution identical for both proteins. A detailed examination reveals two patterns of PIEZO2 immunostaining: cytoplasmic with perinuclear halo (**e**,**f**) and dot-like (**g**,**h**). Scale bars 100 μm (**a**,**c**,**e**,**g**), 50 μm (**b**,**d**), 20 μm (**f**,**h**).

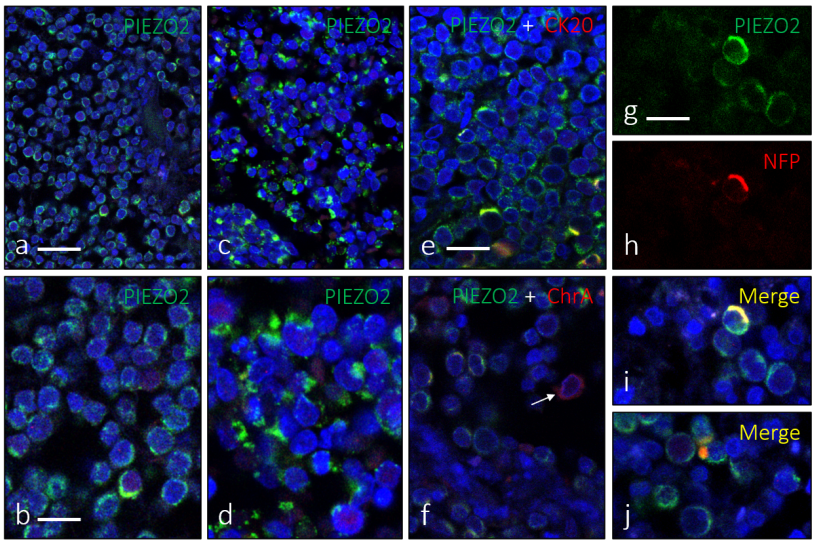

Figure 3. Immunofluorescence for PIEZO2, showing its two morphological patterns: cytoplasmic perinuclear (**a**,**b**) and dot-like pattern (**c**,**d**). Co-localization of PIEZO2 with CK20 (**e**) or ChrA (**f**) was regularly observed (merge in yellow). Arrow in 'f' indicates a ChrA+/PIEZO2− cell. The MCC cells were processed for simultaneous detection of PIEZO2 and NFP (**g**–**j**) only revealed a scarce number of cells showing co-localization of both (**i**), sometimes resembling MC-nerve contact (arrow in **j**). Scale bars 50 μm (**a**,**c**), 20 μm (**b**,**d**–**j**).

PIEZO2 immunoreactivity was also identified employing immunonofluorescence (Figure 3). Using double immunohistochemistry for PIEZO2 and CK20 (Figure 3e), PIEZO2 and ChrA (Figure 3f), or PIEZO2 and NFP (Figure 3g–j), we observed co-localization of those proteins in a variable proportion of cells within the tumor mass.

In addition to MC of the basal epidermal layer and the neoplastic cell mass of MCC, PIEZO2 immunoreactivity was detected in some other cutaneous structures, primarily in ducts of sweat and sebaceous glands. It was observed in nearly all ductal superficial cells and not in basal epithelial elements, morphologically identified as myoepithelial cells (Figure 4a,d). Furthermore, these PIEZO2-positive cells, in contrast with most cells in MCC, were in contact with nerve profiles (Figure 4b,c,e,f).

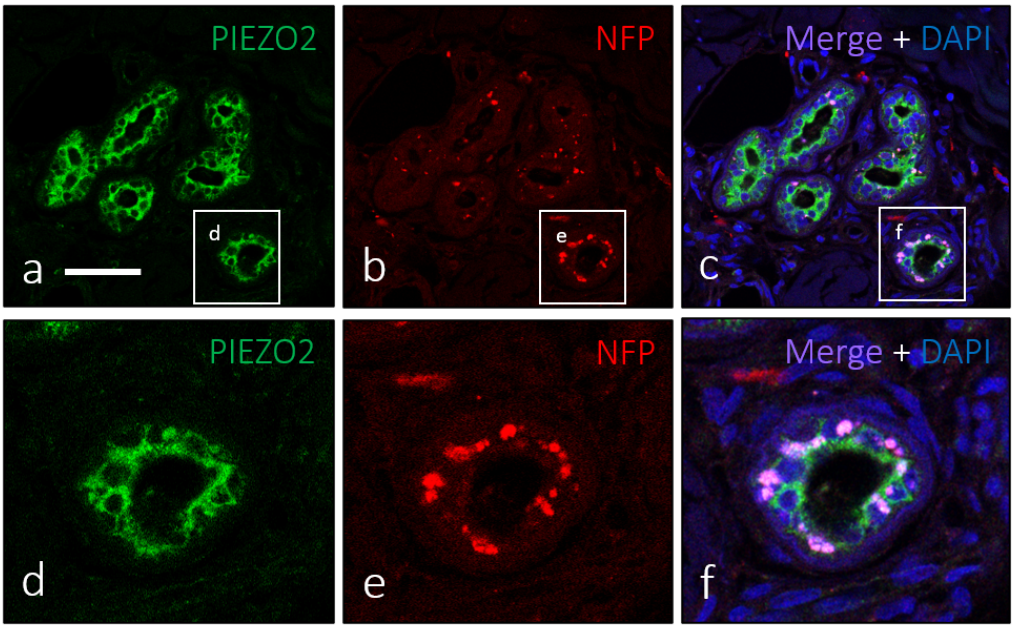

Figure 4. The ductal cells of sweat glands show PIEZO2 immunostaining (**a**,**d**) and were densely innervated (**b**,**c**,**e**,**f**). Scale bar 100 µm.

4. Discussion

Although it has been definitively established that MCs are specialized epithelial cells [29,30], they display a mixed immunohistochemical profile proper of epithelial and neuroendocrine cells [2]. The common ectodermal origin of both neuroendocrine and epidermal lineages might explain that mixed phenotype. Here, we demonstrate that, similarly to MCs, MCC display PIEZO2 immunoreactivity, with a similar dot-like pattern presented by other antigens such as intermediate filaments, typical of neuroendocrine malignancies [10,31]. The expression of this MCs essential protein is another of the many features shared by MCs and MCC cells. To the best of our understanding, the occurrence of PIEZO proteins in MCC was never demonstrated, although it was reported in human MCC-13 cell line [27].

In neoplastic tissue, the role of PIEZO channels, in particular PIEZO2, is still under study [15]. Furthermore, both extracellular matrix and intracellular cytoskeleton are involved in PIEZO2 function in addition to neoplastic cells [16]. The stroma is implicated indirectly, by maintaining the electrochemical gradient necessary for $Ca2^{+}$ influx (for example, K2P, KCa channels) [28]. In addition, the precise composition of the cellular lipid bilayer may be relevant for mechanotransduction [16]. Mechanical forces stimulate these channels both pushing and pulling the cell membrane through the extracellular matrix, whose presence is required for a correct function of PIEZO channels [32]. These forces may influence cancer biology by affecting both neoplastic cells and their microenvironment, by altering cell migration, proliferation, extracellular matrix remodelling and metastatic

spread [28]. Moreover, integrin activation on the cell surface and adhesion to other cells and extracellular matrix depends on the presence of PIEZO1 channels [33]. However, although PIEZO channels have emerged as the key element in mechanotransduction, several types of mechanosensitive ion channels are also involved in this ability, and even other channels may be decisive for mechanotransduction in addition to PIEZO [34].

MCC is known for its poor prognosis and aggressive behavior and exhibits not only PIEZO2 expression in the MCC-13 cell line [27], but also widespread expression on all the tumors studied in our work. PIEZO2 could be significant in the progression of MCC, but if this has clinical relevance or is a mere remnant of the Merkel cell where the MCC developed, remains to be demonstrated in future studies using larger series searching for PIEZO2-negative MCC cases, to determine if this protein define a subgroup of MCC with different clinical or molecular characteristics. In any case, although in the presented MCC cases there is no evidence of a prognostic value of PIEZO2, MCC could benefit from a possible PIEZO related therapy, as long as both types of PIEZO channels are known to be inhibited by the toxin GsMTx4 and stimulated by Yoda1 [34]. Other promising therapies are in study or already in use, such as PD-1/PDL-1 pathway blocking agents, multi-targeted tyrosine kinase inhibitors, MCPyV-related immunotherapy and AURKA inhibitors, potentially useful in MCPyV-negative MCC, among others [9,10,35,36].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jpm12060894/s1, Figure S1: characterization of normal epidermal Merkel cells.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Complejo Asistencial Universitario de Salamanca (protocol code CEIm PI 2022 02 935, approved the 25 April 2022).

Informed Consent Statement: The patients were completely anonymized when obtaining the consent was impossible, due to the time passed since their diagnosis.

Data Availability Statement: Not applicable.

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4. Discusión

4. Discusión

4.1. Consideraciones generales

El presente trabajo de tesis doctoral forma parte de una de las líneas de investigación que durante 40 años se viene desarrollando en el Área de Anatomía y Embriología Humana del Departamento de Morfología y Biología Celular y en los últimos 10 años en el marco del grupo SINPOS (Sistema Nervioso periférico y órganos de los sentidos) del clúster de Biomedicina del Campus de Excelencia internacional de la Universidad de Oviedo.

La línea de investigación principal de este grupo se centra en el estudio de las formaciones sensitivas relacionadas con el tacto. Recientemente, los estudios sobre esta modalidad sensorial han logrado un notable interés que se ha visto reconocido con la concesión del Premio Nóbel de Medicina y Fisiología de 2021, a Ardem Patapoutian y David Julius, que describieron la importancia y especificidad de algunas familias de canales iónicos en los diferentes tipos de sensibilidad. En concreto, el grupo de Patapoutian descubrió en 2010 (Coste et al., 2010) una familia de canales iónicos que denominaron Piezo (con dos miembros conocidos hasta la fecha, PIEZO1 y PIEZO2) relacionados con la detección de las distintas modalidades de estímulos mecánicos (presión externa, estiramiento, tacto, etc …). A partir de ese momento empezaron a conocerse los mecanismos moleculares mediante los cuales los estímulos mecánicos pueden generar impulsos nerviosos. Los estudios sobre modelos murinos han demostrado que PIEZO2 juega un papel crucial en la sensación táctil inocua (Ranade et al., 2014), la propiocepción (Woo et al., 2015) y la nocicepción mecánica (Murthy et al., 2018). Las mismas funciones están mediadas por PIEZO2 en humanos. En los últimos años se ha observado que las mutaciones en *Piezo2* determinantes de una pérdida de función en PIEZO2 presentan déficits en la discriminación del tacto y propiocepción articular y no desarrollan alodinia mecánica (Chesler et al., 2016; Haliloglu et al., 2017; Szczot et al., 2018; Masingue et al., 2019; Yamaguchi et al., 2019; Oakley-Hannibal et al., 2020).

Los datos disponibles en la actualidad establecen, de forma definitiva, que PIEZO2 es clave en el mecanismo de mecanotransducción y, por tanto, esencial en la mecanosensación del tacto fino, el tacto discriminatorio, la sensación de presión, el estiramiento y la vibración. En base a ello se ha organizado el presente trabajo de tesis doctoral analizando mediante técnicas de inmunohistoquímica la presencia de PIEZO2 en las formaciones mecanosensitivas cutáneas humanas de diferentes localizaciones anatómicas.

Actualmente también se conoce que PIEZO2 media la mecanosensibilidad de algunos tejidos no nerviosos y está implicado en la etiopatogenia de algunas enfermedades y diversos tipos de cáncer. Por ello se han realizado algunos experimentos, incluidos en la tesis, sobre la detección de PIEZO2 en tejidos no nerviosos, en también en condiciones de patología sistémica como la diabetes, y en un tipo de tumor que, en buena lógica, debe expresar este canal: el carcinoma de células de Merkel.

En los párrafos que siguen se realizará una discusión global de los resultados, ya que en cada uno de los trabajos que constituyen la tesis se ha realizado una discusión específica.

4.2. Discusión de los resultados

4.2.1 La sinaptofisina es un marcador selectivo de axones en los órganos sensitivos complejos cutáneos humanos y se colocaliza con PIEZO2

Aunque existen diferentes marcadores estructurales de los axones (neurofilamentos, periferina) y citosólicos (enolasa neuronal específica y la proteína PGP 9.5) algunos resultados previos de nuestro laboratorio sugerían que la sinaptofisina, una proteína de las vesículas sinápticas, también podría ser un buen marcador. En nuestra investigación hemos observado que se localiza en los axones que suplen los órganos sensitivos cutáneos, en los husos neuromusculares y en una subpoblación neuronal sensitiva que cumple los requisitos para ser considerada como mecanoceptora. Además, en el caso de los corpúsculos de Meissner, de Pacini y en los complejos células de Merkel-axón se

colocaliza con la mecanoproteína PIEZO2. En trabajos próximos analizaremos si también se localiza en las motoneuronas y en las neuronas y fibras vegetativas postganglionares. Los resultados obtenidos abren nuevas vías de investigación ya que, con la excepción de Vargas et al. (2020) que describe la presencia de sinaptofisina en el axón de los corpúsculos de Pacini, nunca se había descrito en los mecanorreceptores. Es posible que se localice en las vesículas claras propias de los terminales axónicos de los corpúsculos sensitivos (Malinosky y park, 1982; Zelen , 1994), pero son necesarios estudios de inmunocitoquímica para confirmarlo.

En las células de Merkel la sinaptofisina se localiza en el polo axónico de las sinapsis-like que se forman entre los axones LTMRs y las propias células. A ese nivel se localizan distintos neurotransmisores que participan en la génesis del potencial de acción célula de Merkel-axón. Pero ese no es el caso de las células gliales terminales del resto de los órganos sensitivos complejos cutáneos. Por tanto, el papel de la sinaptofisina en la mecanosensibilidad y la mecanotransducción debe ser abordado en futuros estudios.

4.2.2 Las células de Merkel y los corpúsculos de Meissner en la piel glabra expresan PIEZO2 con independencia de su localización anatómica

El trabajo original describiendo la distribución de PIEZO2 la detectó, como era de esperar en base a la función propuesta, en los mecanorreceptores y en los terminales cutáneos de estos: la formaciones cutáneas complejas, es decir los corpúsculos sensitivos, y en las células de Merkel. Nuestro grupo, en una serie de estudios, pudimos observar que, como regla general, las células de Merkel, y el axón que suple los corpúsculos de Pacini y de Meissner son PIEZO2 positivos. Nuestro grupo de trabajo fue el primero en demostrar la presencia de PIEZO2 en los corpúsculos sensitivos humanos ampliando estudios iniciales en la piel glabra del ratón.

Este patrón de distribución lo hemos descrito en la piel digital, la piel del clítoris y del prepucio, y en la piel de la areola mamaria y el pezón (Gutiérrez-Villanueva et al., 2020). En otras zonas anatómicas cubiertas de piel glabra como los labios (Martín-Cruces et al.,

datos no publicados), los resultados preliminares también sugieren la presencia de PIEZO2 en las formaciones sensitivas de estas estructuras.

En los vertebrados, la mecanotransducción ocurre en órganos sensitivos especializados llamados clásicamente corpúsculos sensitivos (Vega et al., 2009; McGlone y Reilly, 2010) y más recientemente formaciones sensitivas cutáneas complejas (Handler y Ginty, 2021). Además, son mecanorreceptores aceptados los complejos célula de Merkel-axón. No debe resultar extraño pues, que si PIEZO2 desempeña un papel clave en la mecanotransducción se detecte en las estructuras anatómicas donde esta tiene lugar.

PIEZO2 está presente en el citoplasma de las células de Merkel, pero no en todas. Esta heterogeneidad de expresión no parece guardar relación con la inervación, ya se observaron fibras nerviosas en contacto tanto con células de Merkel PIEZO2 positivas como negativas. Además, se debe subrayar que las fibras nerviosas de los complejos células de Merkel-neurita siempre fueron PIEZO2 negativas. Resulta difícil de explicar por qué mientras los axones de los corpúsculos son PIEZO2 positivos los que contactan con las células de Merkel no lo son; este asunto queda pendiente para ser investigado en posteriores trabajos.

Por otro lado, en los estudios sobre la distribución de PIEZO en los terminales cutáneos de los mecanorreceptores obtuvimos las primeras evidencias de que esta mecanoproteína también se encuentra en los tejidos no nerviosos: epidermis, endotelio de los vasos y glándulas sudoríparas (ver más adelante); su detección en tejidos epiteliales estaba en línea con lo publicado previamente por nuestro grupo de investigación que lo detecto en la mama (Gutiérrez-Villanueva et al., 2020).

4.2.4 Los mecanorreceptores cutáneos adquieren mecanocompetencia antes del nacimiento

Un aspecto casi completamente desconocido del sentido del tacto es cuándo se adquiere a nivel periférico, es decir, cuándo las formaciones nerviosas sensitivas cutáneas son capaces de mecanotransducir. Para tratar de responder a esta pregunta

en el presente trabajo de tesis doctoral se ha considerado la expresión de PIEZO2 por los axones de los corpúsculos y por las células de Merkel como evidencia de que los mecanorreceptores son funcionantes. De forma paralela se completó el trabajo previo de Feito et al. (2018) sobre el desarrollo de las formaciones mecanosensibles cutáneas estudiando el desarrollo de las células de Merkel cutáneas mediante marcadores específicos.

Aunque se encontraron evidencias de células potencialmente identificables como células de Merkel a las 13 semanas wega, solo a partir de las semanas 22-23 wega se identificaron células típicas que aumentan en densidad hasta la tercera semana postnatal; después disminuyen de forma brusca y se mantienen estables hasta los 20 años de vida. En concomitancia con la adquisición de la morfología definitiva las células de Merkel adquieren comienzan a expresar PIEZO2 y continúan siendo positivas para esta mecanoproteína durante todo el periodo de vida analizado. Por otra parte, puede considerarse que las células de Merkel PIEZO2+ son mecanotransductoras porque están inervadas, aunque como ya se comentó previamente los axones que las inervan son mayoritariamente PIEZO2 negativos.

En el presente estudio también utilizó la presencia de PIEZO2 en los corpúsculos de Meissner y Pacini como evidencia de que son funcionalmente activos. En ambos casos, desde la 11 wega del periodo prenatal hasta los 20 años de vida los axones de ambos tipos de formaciones nerviosas sensitivas son mecanotransductoras. Este trabajo viene a rellenar un periodo de tiempo no estudiado previamente y que completa estudios previos de nuestro grupo de investigación durante la edad adulta y la senescencia (García-Mesa et al., 2017; García-piqueras et al., 2019).

4.2.5 Los corpúsculos sensitivos cutáneos en la neuropatía dolorosa y no dolorosa: implicación del PIEZO2

La neuropatía diabética cursa con un deterioro progresivo del sistema somatosensorial que implica desde los receptores cutáneos hasta la corteza cerebral (Vinik et al., 2015; Shilo et al., 2019; Rosenberger et al., 2020). El dolor, cuando aparece, es de tipo

neuropático (Lauria et al., 2012; Themistocleous et al., 2016; Shillo et al., 2019; Rosenberger et al., 2020) y *a priori* los corpúsculos sensitivos no deberían estar involucrados neuropatía diabética. Por ello se decidió analizar sin existe afectación en los formaciones sensitivas cutáneas en la neuropatía diabética, y si existen diferencias entre los sujetos con dolor y sin dolor.

Los estudios en modelos animales (Paré et al., 2007) y en humanos (Peltier et al., 2013) han demostrado que la diabetes produce una reducción de la densidad y alteraciones estructurales en los corpúsculos sensitivos. Nuestros hallazgos coinciden con los de estos autores en el casos de neuropatía no dolorosa, y es más evidente en los casos de neuropatía no dolorosa. En otras palabras, la evolución de la neuropatía diabética de no dolorosa a dolorosa se acompaña de cambios progresivos en los corpúsculos sensitivos cutáneos que sugieren una denervación y atrofia de los mismos. Además, como consecuencia de la disrupción de los axones en la neuropatía diabética, especialmente la dolorosa, se pierde la expresión de las proteínas que intervienen directa o indirectamente en la mecanotransducción, es decir PIEZO2.

Por tanto, la pérdida de los mecanotransductores cutáneos, así como de las mecanoproteínas presentes en ellos, rompería el control de la sensibilidades cutáneas a nivel del asta posterior de la médula espinal (teoría de la *gate control*) dando origen a la aparición del dolor. Esta atractiva hipótesis debe ser confirmada en posteriores estudios.

4.2.6 PIEZO2 en tejidos no nerviosos: el ejemplo del aparato urogenital

Mecanotransducción, como se viene reiterando a lo largo de este trabajo, es la conversión de los estímulos mecánicos en efectos biológicos, químicos o físicos. Según este concepto, resulta evidente que la mayoría de las células pueden responder a la acción de las fuerzas.

Aunque las mecanoproteínas, especialmente PIEZO2, se descubrieron en el sistema nervioso periférico aferente, los estudios posteriores han demostrado que tienen una

distribución mucho más amplia que incluye a muchos tejidos no nerviosos. Ello no debe de extrañar si se tiene en cuenta que el pulmón, por ejemplo, detecta la variaciones en la presión parcial del aire; o que el tubo digestivo es sensible a los cambios que se producen en la distensión de sus paredes. En nuestro laboratorio se está llevando a cabo en la actualidad un estudio sistemático de mapeo mediante inmunohistoquímica de la expresión de PIEZO2 en todos los órganos del cuerpo. Algunas partes ya están completamente finalizadas y en los próximos meses se someterán para su publicación. En este trabajo de tesis doctoral se han incluido, exclusivamente, los resultados en el sistema urogenital: aparato urinario y aparatos genital masculino y genital femenino. Algunas publicaciones recientes han detectado mediante inmunohistoquímica PIEZO1 en el riñón y vías urinarias, así como en vagina, próstata, vesículas seminales y conductos eyaculatorios del ratón (Dalghi et al., 2019). Por otro lado, PIEZO2 se encontró en el riñón y vejiga de la orinas de ratón y humanos (Etem et al., 2018; Coste et al., 2010; Yang et al., 2016).

Además de en las vías urinarias y riñón, nuestro estudio demuestra que también está presente en otros órganos como el testículo, el ovario, la tubas uterinas y te propio útero. Aunque aún se está lejos de conocer en detalle las funciones de estas mecanoproteínas en los órganos en los que se han detectado, algunos trabajos sugieren que PIEZO1 regula la osmolaridad de la orina (Martins et al., 2016) mientras que PIEZO2 participaría en la regulación del flujo urinario y en la detección de la distensión vesical (Scholz et al., 2016; Coste et al., 2010; Murthy et al., 2017; Geng et al., 2017; Marshall et al., 2020). Y por otro lado, en base a los resultados obtenidos en los órganos genitales no es de descartar que intervengas en los procesos de espermatogénesis y ovogénesis, pero sobre todo en los procesos en que la mecanobiología sea transcendente como en la eyaculación, transporte el zigoto por la tuba o el acoplamiento del útero a la gravidez.

4.2.7 Los tumores derivados de las células de Merkel son PIEZO2 positivos

Las características únicas de los canales de la familia PIEZO, su funcionalidad mecanosensitiva y disposición como proteínas de transmembrana, hicieron pensar desde el principio que su expresión podría estar alterada en el cáncer, sobre todo en los

tipos tumorales propensos a un alto grado de estrés mecánico. Esta hipótesis nace del hecho de que la eliminación de PIEZO1 y PIEZO2 favorece la migración celular *in vitro* y el crecimiento del tumor *in* vivo (Ra nade et al., 2015; Woo et al., 2015). Existen datos de que PIEZO1 se sobreexpresa en el cáncer de mama (Li et al., 2015), gástrico (Zhang et al., 2018), carcinoma de vejiga (Etem et al., 2018), osteosarcoma (Jiang et al., 2017) y sarcoma sinovial (Suzuki et al., 2018), cáncer de próstata (Han et al., 2019), carcinoma oral (Hasegawa et al., 2021). Por el contrario, la pérdida de funcionalidad de PIEZO1 se observó en el cáncer de pulmón (Gyorffy et al., 2013). Por lo que respecta a PIEZO2, la sobreexpresión se asocia al carcinoma de vejiga (Etem et al., 2018) y aumenta la capacidad de invasión en el cáncer de mama triple negativo (Katsuta et al., 2022). Además, se le atribuye una implicación directa en la angiogénesis tumoral (Yang et al., 2016)

El carcinoma de célula de Merkel (CCM) o merkeloma, es un tumor neuroendocrino agresivo con alta propensión a la propagación (Ramahi et al., 2013; Becker et al., 2017; Harms et al., 2017), siendo letal en el 3% de los casos (Hodgson et al., 2017). Se presenta en individuos de avanzada edad en los que la exposición al sol y la inmunosupresión son los factores de riesgo (Cook et al., 2019). Las células de Merkel son células epiteliales altamente especializadas localizadas en la capa basal de la epidermis, que forman parte de los complejos célula de Merkel-neurita. Se ha demostrado en los apartados anteriores de esta tesis, que tanto las neuronas sensitivas periféricas como las células de Merkel, son positivas para el canal PIEZO2 en la piel glabra digital humana; y que la densidad de las células de MC, como la inmunorreacción positiva para PIEZO2 disminuyen con la edad (García-Mesa 2017; García-Mesa et al., 2022), lo que contrasta con el hecho de que los CCM se presentan en los ancianos.

Las fuerzas mecánicas estimulan a estos canales, tanto empujando como tirando de la membrana celular a través de la matriz extracelular (Gaub y Muller, 2017) y ello condiciona la biología del cáncer, actuando sobre las células neoplásicas y el microambiente, lo que provoca efectos sobre la migración celular, la proliferación, la remodelación de la matriz y la diseminación metastásica (Petho et al., 2019). La expresión de PIEZO2 en el CCM no se ha investigado previamente, aunque la línea

celular MCC-13 (línea de carcinoma de Merkel humano) es positiva para este canal (Shin et al., 2019). Este trabajo muestra por primera vez la presencia de este canal en los citoplasmas de las células neoplásicas de todos los merkelomas estudiados; a nivel diagnóstico, el CCM se identificaba por la inmunorreacción positiva para los anticuerpos CK20, Cromogranina A, SYN o NFP. Es posible que la presencia de PIEZO2 en los CCM esté relacionada con su progresión y desarrollo del mismo, aunque se deben realizarse más estudios con el fin de comprobar si existen CCM negativos, y definir si la expresión de esta proteína define un subgrupo con diferentes características clínicas y moleculares.

4.3. Limitaciones del estudio y perspectivas de futuro

Con carácter general se han completado con éxito todo los objetivos propuestos, respondiendo a las preguntas de investigación que se plantearon en cada uno de ellos. No obstante, a lo largo del desatollo del trabajo de la tesis han surgido nuevas dudas que serán abordadas en próximos proyectos del grupo SINPOS.

Un resultado que merecerá nuestro interés es trata de esclarecer por qué existen variaciones en los componentes de los LTMRs en función del morfotipo: así, mientras PIEZO2 está presente en los axones los corpúsculos de Meissner y Pacini, y ausente de las células auxiliares gliales terminales, en los complejos neurita-célula sucede lo contrario, es decir, las Merkel son positivas y los axones que las inervan negativos.

En los próximos meses se procederá a completar los estudios ya en marcha encaminados a realizan un mapeo completo de las proteínas PIEZO1 y PIEZO2 en todos los tejidos humanos. Se trata de ofrecer un estudio de base que sirva para analizar la posible funcionalidad de ambas proteínas en condiciones de normalidad y patológicas, sobre todo en patologías inflamatorias y en el cáncer. Es demasiado pronto para especular sobre posibles terapias basadas en interacciones con mecanoproteínas de la familia Piezo, pero disponer de mapeos de expresión puede ser un buen comienzo.

5. Conclusiones

Tras el análisis detallado de los resultados y la oportuna discusión de los mismos con los datos disponibles, se ha llegado a las siguientes conclusiones:

1.- En los órganos sensitivos cutáneos complejos considerados como LTMRs de adaptación rápida (corpúsculos de Meissner y relacionados y corpúsculos de Pacini) el axón expresa PIEZO2 con independencia de su localización anatómica (piel digital, del clítoris y del glande).

2.- En los órganos sensitivos cutáneos complejos considerados como LTMRs de adaptación lenta (complejos célula de Merkel-neurita) los axones que los forman son PIEZO2 negativos mientras que las células accesorias (células de Merkel son PIEZO2 positivas) con independencia de su localización anatómica. Esta conclusión no ha podido verificarse en el caso de los corpúsculos de Ruffini.

3.- En base a la expresión de PIEZO2 las células de Merkel de la piel digital humana adquieren mecanocompetencia a las semanas 22-23 semanas de edad de gestación estimada, y los corpúsculos de Meissner y Pacini a partir de las 11 semanas de edad de gestación estimada.

4.- En el riñón y vías urinarias humanas, así como en los diferentes órganos de los aparatos genitales femenino y masculino, se detectaron poblaciones de células PIEZO2 (y PIEZO1) positivas, en su mayoría de estirpes epiteliales.

5.- La neuropatía diabética en su progresión de no dolorosa a dolorosa cursa con un deterioro progresivo de la estructura de los corpúsculos sensitivos de la pile glabra humano, consistente con denervación, acompañado de la expresión de proteínas mecanosensibles.

6.- Las células del carcinoma de células de Merkel con PIEZO2 positivas.

6. Bibliografía

6. Bibliografía

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Yang H, Liu C, Zhou RM, Yao J, Li XM, Shen Y, Cheng H, Yuan J, Yan B, Jiang Q. Piezo2 protein: A novel regulator of tumor angiogenesis and hyperpermeability. Oncotarget. 2016; 7(28):44630-44643. doi: 10.18632/oncotarget.10134.

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Anexo 1 – Curriculum vitae

MINISTERIO
DE EDUCACIÓN,
CULTURA Y DEPORTE

SECRETARÍA DE
ESTADO DE
EDUCACIÓN

DIRECCIÓN GENERAL
DE UNIVERSIDADES

COMISIÓN NACIONAL EVALUADORA
DE LA ACTIVIDAD INVESTIGADORA

Currículum vitae

Impreso normalizado

Número de hojas que contiene: 11

Nombre: YOLANDA GARCÍA MESA

Fecha: 18.10.2022

Firma:

El arriba firmante declara que son ciertos los datos que figuran en este currículum, asumiendo en caso contrario las responsabilidades que pudieran derivarse de las inexactitudes que consten en el mismo.

No olvide que es necesario firmar al margen cada una de las hojas

Este currículum no excluye que en el proceso de evaluación se le requiera para ampliar la información aquí contenida.

1. INFORMACIÓN PERSONAL

APELLIDOS: GARCÍA MESA NOMBRE: YOLANDA

SEXO: MUJER DNI: FECHA DE NACIMIENTO:

DIRECCION PARTICULAR:

CIUDAD: CODIGO POSTAL: ESPECIALIZACIÓN: TELEFONO:

ENFERMERA

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2. FORMACIÓN ACADÉMICA

1. GRADO: ENFERMERÍA

CENTRO: FACULTAD DE ENFERMERÍA Y FISIOTERAPIA. UNIVERSIDAD DE OVIEDO

FECHA: 2012-2016

2. MÁSTER: INVESTIGACIÓN E INNOVACIÓN EN NEUROCIENCIAS

CENTRO: UNIVERSIDAD DE OVIEDO

FECHA: 2016-2017

3. MÁSTER: INTEGRACIÓN EN CUIDADOS Y RESOLUCIÓN DE PROBLEMAS CLÍNICOS EN ENFERMERÍA

CENTRO: UNIVERSIDAD DE ALCALÁ

FECHA: 2016-2017

4. CAPACITACIÓN CUIDADOS CRÍTICOS:

CENTRO: HOSPITAL DE CABUEÑES

FECHA: 2017-2018

5. DOCTORADO: CIENCIAS DE LA SALUD (SISTEMA NERVIOSO PERIFÉRICO Y ÓRGANOS DE LOS SENTIDOS). CURSANDO ACTUALMENTE (2017-ACTUALIDAD). DIRECTOR DE TESIS: JOSE ANTONIO VEGA ÁLVAREZ / OLIVIA GARCÍA SUÁREZ

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3. SITUACIÓN PROFESIONAL ACTUAL

ORGANISMO: UNIVERSIDAD DE OVIEDO

FACULTAD, ESCUELA o INSTITUTO DEL C.S.I.C.: FACULTAD DE MEDICINA

DEPT./SECC./ UNIDAD ESTR.: DEPARTAMENTO MORFOLOGÍA Y BIOLOGÍA CELULAR

CATEGORIA PROFESIONAL Y FECHA DE INICIO: BECARIA SEVERO OCHOA (2018)

DIRECCION POSTAL: 33006

TELEFONO (indicar prefijo, número y extensión): 664714710

4. ACTIVIDADES ANTERIORES DE CARACTER CIENTIFICO O PROFESIONAL

FECHAS	PUESTO	NSTITUCIÓN
01/07/2016-30/08/2016	ENFERMERA	HOSPITAL DE JARRIO
02/08/2017-30/08/2017	ENFERMERA	HOSPITAL DE JARRIO
2016-2017	BECARIA	FACULTAD MEDICINA OVIEDO
2017-2018	COLAB. HONOR.	FACULTAD MEDICINA OVIEDO
2018-ACTUALIDAD.	BECARIA	FACULTAD MEDICINA OVIEDO
2019-2022	DOCENCIA EN ANATOMÍA (BECA)	FACULTAD MEDICINA OVIEDO

5. PARTICIPACION EN PROYECTOS DE INVESTIGACION FINANCIADOS

1. TITULO DEL PROYECTO: LA BIOPSIA CUTÁNEA DE LOS CORPÚSCULOS DE MEISSNER PARA EL DIAGNÓSTICO DE LA NEUROPATÍA DIABÉTICA

ENTIDAD FINANCIADORA: MINISTERIO DE EDUCACIÓN, CULTURA DE EDUCACIÓN, CULTURA Y DEPORTE.

DURACION DESDE: 2016 HASTA:2017

INVESTIGADOR PRINCIPAL: YOLANDA GARCÍA MESA

2. TITULO DEL PROYECTO: ACTUACIÓN PARA EL SEGUIMIENTO DE LA ADQUISICIÓN DE CONOCIMIENTOS POR PARTE DE LOS ALUMNOS DE LA ASIGNATURA DE ANAATOMÍA HUMANA EN EL GRADO DE ENFERMERÍA

ENTIDAD FINANCIADORA: UNIVERSIDAD DE OVIEDO

DURACION DESDE: 2020 HASTA:2021

INVESTIGADOR PRINCIPAL: OLIVIA GARCÍA SUÁREZ Y YOLANDA GARCÍA MESA

PROYECTO DE INNOVACIÓN DOCENTE

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6. PUBLICACIONES

1. García-Mesa Y, García-Piqueras J, García B, Feito J, Cabo R, Cobo J, Vega JA, García-Suárez O. Merkel cells and Meissner's corpuscles in human digital skin display Piezo2 immunoreactivity. J Anat. 2017 Dec;231(6):978-989. doi: 10.1111/joa.12688. Epub 2017 Sep 14. PubMed PMID: 28905996; PubMed Central PMCID: PMC5696134. Q1
2. Feito J, García-Suárez O, García-Piqueras J, García-Mesa Y, Pérez-Sánchez A, Suazo I, Cabo R, Suárez-Quintanilla J, Cobo J, Vega JA. The development of human digital Meissner's and Pacinian corpuscles. Ann Anat. 2018 Sep; 219:8-24. doi:10.1016/j.aanat.2018.05.001. Epub 2018 May 26. PubMed PMID: 29842990. Q2
3. Feito J, Cebrián-Muiños C, Alonso-Morrondo EJ, García-Mesa Y, García-Piqueras J, Cobo R, García-Suárez O, Vega JA. Hyperplastic sensory corpuscles in nevus sebaceus of labia minora pudendi. A case report. J Cutan Pathol. 2018 Oct;45(10):777-781. doi: 10.1111/cup.13316. Epub 2018 Aug 3. PubMed PMID:29961991. Q2
4. García-Piqueras J, Carcaba L, García-Mesa Y, Feito J, García B, Viña E,Suárez-Quintanilla J, Cobo J, Vega JA, García-Suárez O. Chondroitin Sulfate in Human Cutaneous Meissner and Pacinian Sensory Corpuscles. Anat Rec (Hoboken). 2019 Feb;302(2):325-331. doi: 10.1002/ar.23951. Epub 2018 Nov 19. PubMed PMID:0299593. Q2
5. García-Piqueras J, García-Mesa Y, Cárcaba L, Feito J, Torres-Parejo I,Martín-Biedma B, Cobo J, García-Suárez O, Vega JA. Ageing of the somatosensory system at the periphery: age-related changes in cutaneous mechanoreceptors. J Anat. 2019 Jun;234(6):839-852. doi: 10.1111/joa.12983. Epub 2019 Mar 29. PubMed PMID: 30924930. Q1

6. García-Piqueras J, García-Mesa Y, Feito J, García B, Quiros LM, Martín-Biedma B, Cobo T, Vega JA, García-Suárez O. Class I and Class II small leucine-rich proteoglycans in human cutaneous pacinian corpuscles. Ann Anat. 2019 Jul;224:62-72. doi: 10.1016/j.aanat.2019.02.007. Epub 2019 Apr 18. PubMed PMID: 31005573. Q2
7. García-Piqueras J, García-Suárez O, García-Mesa Y, García-Fernandez B, Quirós LM, Cobo R, Martín-Biedma B, Feito J, Vega JA. Heparan sulfate in human cutaneous Meissner's and Pacinian corpuscles. Anat Rec (Hoboken). 2019 Dec 9. doi: 10.1002/ar.24328. [Epub ahead of print] PubMed PMID: 31815364. Q2
8. García-Piqueras J, Cobo R, Cárcaba L, García-Mesa Y, Feito J, Cobo J, García-Suárez O, Vega JA. The capsule of human Meissner corpuscles: immunohistochemical evidence. J Anat. 2019 Dec 22. doi: 10.1111/joa.13139. [Epub ahead of print] PubMed PMID: 31867731. Q1
9. Gutiérrez-Villanueva M, García-Mesa Y, García-Piqueras J, Cobo R, García-Suárez O, Vega JA, Feito J. The sensory innervation of the human nipple. Ann Anat. 2020 Jan 3;229:151456. doi: 10.1016/j.aanat.2019.151456. [Epub ahead of print] PubMed PMID: 31911160. Q2
10. García-Suárez O, García-Mesa Y, García-Piqueras J, Salvo G, Cobo JL, Alba E, Cobo R, Feito J, Vega JA. (November 5th 2018). The Cutaneous Biopsy for the Diagnosis of Peripheral Neuropathies: Meissner's Corpuscles and Merkel's Cells [Online First], IntechOpen, DOI: 10.5772/intechopen.81687. Available from: https://www.intechopen.com/online-first/the-cutaneous-biopsy-for-the-diagnosis-of- peripheral-neuropathies-meissner-s-corpuscles-and-merkel-s. (CAPÍTULO DE LIBRO).
11. García-Mesa Y, Cárcaba L, Coronado C, Cobo R, Martín-Cruces J, García-Piqueras J, Feito J, García-Suárez O, Vega JA. Glans clitoris innervation: PIEZO2 and sexual mechanosensitivity. J Anat. 2021 Feb;238(2):446-454. doi: 10.1111/joa.13317. Epub 2020 Sep 29. PMID: 32996126; PMCID: PMC7812125. Q1
12. Cobo R, García-Mesa Y, Cárcaba L, Martin-Cruces J, Feito J, García-Suárez O, Cobo J, García-Piqueras J, Vega JA. Verification and characterisation of human digital Ruffini's sensory corpuscles. J Anat. 2021 Jan;238(1):13-19. doi: 10.1111/joa.13301. Epub 2020 Aug 31. PMID: 32864772; PMCID: PMC7754963. Q1
13. García-Mesa Y, García-Piqueras J, Cobo R, Martín-Cruces J, Suazo I, García-Suárez O, Feito J, Vega JA. Sensory innervation of the human male prepuce: Meissner's corpuscles predominate. J Anat. 2021 Oct;239(4):892-902. doi: 10.1111/joa.13481. Epub 2021 Jun 12. PMID: 34120333; PMCID: PMC8450466. Q1

14. García-Mesa Y, Feito J, González-Gay M, Martínez I, García-Piqueras J, Martín-Cruces J, Viña E, Cobo T, García-Suárez O. Involvement of Cutaneous Sensory Corpuscles in Non-Painful and Painful Diabetic Neuropathy. J Clin Med. 2021 Oct 8;10(19):4609. doi: 10.3390/jcm10194609. PMID: 34640627; PMCID: PMC8509589. Q1
15. Pastor JF, Muchlinski MN, Potau JM, Casado A, García-Mesa Y, Vega JA, Cabo R. The Tongue in Three Species of Lemurs: Flower and Nectar Feeding Adaptations. Animals (Basel). 2021 Sep 27;11(10):2811. doi: 10.3390/ani11102811. PMID: 34679832; PMCID: PMC8532830. Q1
16. Solis-Hernandez MP, Martín C, García B, Pérez-López N, García-Mesa Y, González-Fernández S, García-Suárez O, Merayo J, Fernández-Vega I, Quirós LM. The Genes Encoding Small Leucine-Rich Proteoglycans Undergo Differential Expression Alterations in Colorectal Cancer, Depending on Tumor Location. Cells. 2021 Aug 6;10(8):2002. doi: 10.3390/cells10082002. PMID: 34440771; PMCID: PMC8391422. Q1
17. Cárcaba L, García-Piqueras J, García-Mesa Y, Cobo R, García-Suárez O, Feito J, Vega JA. Human digital merkel cells display pannexin1 immunoreactivity. Ann Anat. 2022 Jan;239:151813. doi: 10.1016/j.aanat.2021.151813. Epub 2021 Aug 10. PMID: 34384856. Q1
18. Suazo I, Vega JA, García-Mesa Y, García-Piqueras J, García-Suárez O, Cobo T. The Lamellar Cells of Vertebrate Meissner and Pacinian Corpuscles: Development, Characterization, and Functions. Front Neurosci. 2022 Mar 9;16:790130. doi: 10.3389/fnins.2022.790130. PMID: 35356056; PMCID: PMC8959428. Q1
19. García-Martínez I, García-Mesa Y, García-Piqueras J, Martínez-Pubil A, Cobo JL, Feito J, García-Suárez O, Vega JA. Sensory innervation of the human palmar aponeurosis in healthy individuals and patients with palmar fibromatosis. J Anat. 2022 May;240(5):972-984. doi: 10.1111/joa.13609. Epub 2021 Dec 8. PMID: 34881452; PMCID: PMC9005682. Q1

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7. PARTICIPACION EN CONTRATOS DE INVESTIGACION DE ESPECIAL RELEVANCIA CON EMPRESAS Y/O ADMINISTRACIONES

TITULO DEL CONTRATO: MECANOBIOLOGÍA MEDIADA POR PIEZO 2. PROYECTO PA-18-PF-BP17-044.

EMPRESA/ADMINISTRACIÓN FINANCIADORA: MINISTERIO DE EDUCACIÓN, CULTURA DE EDUCACIÓN, CULTURA Y DEPORTE.

DURACIÓN DESDE: 2018 HASTA:2022

INVESTIGADOR RESPONSABLE: JOSE ANTONIO VEGA ÁLVAREZ

8. CONGRESOS

1. “Evaluation of Meissner corpuscles and Merkel cells in diabetic neuropathy”. (García-Mesa Y, García-Piqueras J, Feito J, Cabo-Pérez R, Vega-Álvarez JA, García-Suárez O). Poster. 52nd Annual Scientific Meeting of European Society for Clinical Investigation. Barcelona, May 30th to June 1st 2018.
2. “Validez de los corpúsculos de Meissner y de las células de Merkel para el diagnóstico de neuropatías en la biopsia cutánea”. Ponencia Oral. (García-Mesa Y, García-Piqueras J, Feito J, Cabo-Pérez R, García-Suárez O, Vega- Álvarez JA). XXVIII Congreso de la Sociedad Anatómica Española. Badajoz (1-3 Febrero de 2018). PONENTE: YOLANDA GARCÍA MESA
3. “Estudio de los corpúsculos de Meissner y las células de Merkel en la esclerosis múltiple y la esclerosis lateral amiotrófica”. Póster. (García-Mesa Y, García-Piqueras J, Feito J, Cabo-Pérez R, Vega-Álvarez JA, García-Suárez O). XXVIII Congreso de la Sociedad Anatómica Española. Badajoz (1-3 Febrero de 2018).
4. “Mecanorreceptores de tacto fino de la piel glabra humana en la neuropatía diabética: estudio inmunohistoquímico de canales de la mecanotransducción”. Ponencia Oral. (García-Mesa Y, García-Piqueras J, Feito J, González-Gay M, García-Martínez I, Viña E, Cabo R, Vega-Álvarez JA, García-Suárez O). 34 Encuentro de Jóvenes Investigadores I.N.I.C.E. 2018. PONENTE: YOLANDA GARCÍA MESA

5. “Angiogenesis and inflammation in the skin of patients with diabetic neuropathy”. Póster. García-Mesa Y, González- Gay M, García-Martínez I, Feito J, Salvo G, Viña E, García-Piqueras J, Cabo R, Vega-Álvarez JA, García-Suárez O. International Congress of Anatomía Clínica (EACA 2019). Madrid. 24-26 Junio 2019.
6. “Localization of the mechanosensitive ion cannel Piezo2 in the human gastrointestinal tract”. Póster. García-Mesa Y, Feito J, Viña E, García-Piqueras J, Ordóñez S, Pérez-Moltó FJ, Vega-Álvarez JA, García-Suárez O. International Congress of Anatomía Clínica (EACA 2019). Madrid. 24-26 Junio 2019.
7. “Cutaneous mechanoreceptors are differently involved in painful and non-painful diabetic neuropathy”. PONENCIA ORAL (PONENTE: YOLANDA GARCÍA MESA). García-Mesa Y, González-Gay M, García-Martínez I, Feito J, García-Piqueras J, Salvo G, Viña E, Cobo R , Vega-Álvarez JA,

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8. "ALTERACIONES EN LA SÍNTESIS DE LA ESTRUCTURA FINA DEL HEPARÁN SULFATO EN EL HIPOCAMPO ANTERIOR DE PACIENTES CON LA ENFERMEDAD DE ALZHEIMER". Natalia Pérez López, Iván Fernández- Vega, María Pilar Solis-Hernandez, Yolanda García Mesa, Víctor Lozano Iturbe, Carla Martín Cueto, Olivia García- Suárez, Luís M. Quirós Fernández y Beatriz García Fernández. XXVIII Congreso Nacional SEAP-IAP, XXIII Congreso Nacional SEC, IV Congreso Nacional SEPAF. (24-26 de Mayo de 2017). Valencia.
9. "THE SENSORY INNERVATION OF THE HUMAN PALMAR APONEUROSIS IN NORMAL CONDITIONS AND IN PALMAR FIBROMATOSIS DISEASE". García-Martínez, Irene, M. Gago, Abel, García-Mesa, Yolanda, Jorge, Feito, Suazo, Iván, García-Suárez, Olivia, Vega, JA. XXIX Congreso de la Sociedad Anatómica Española que tuvo lugar en la Universitat Jaume I y ha sido organizado por la Fundación Universitat Jaume I- Empresa en colaboración con la Sociedad Anatómica Española, celebrado los días 15, 16 y 17 de septiembre de 2021.
10. "DISTRIBUTION OF SENSORY FORMATIONS IN HUMAN SHOULDER JOINTS". M. Gago, Abel; García-Mesa, Yolanda; Garrido, Andrea; Viña, Eliseo; Cabo, Roberto; Muriel, Juan D.; Vega, José A. XXIX Congreso de la Sociedad Anatómica Española que tuvo lugar en la Universitat Jaume I y ha sido organizado por la Fundación Universitat Jaume I- Empresa en colaboración con la Sociedad Anatómica Española, celebrado los días 15, 16 y 17 de septiembre de 2021.
11. "IMMUNOHISTOCHEMICAL LOCALIZATION OF PIEZO1 AND PIEZO2 IN HUMAN URINARY SYSTEM". Yolanda García-Mesa, Jorge Feito, Abel Martínez-Gago, Roberto Cabo, Pérez-Moltó P, Félix de Paz, Marcos Gutiérrez-Villanueva, Olivia García Suárez. XXIX Congreso de la Sociedad Anatómica Española que tuvo lugar en la Universitat Jaume I y ha sido organizado por la Fundación Universitat Jaume I- Empresa en colaboración con la Sociedad Anatómica Española, celebrado los días 15, 16 y 17 de septiembre de 2021.
12. "THE SENSORY INNERVATION OF HUMAN LIPS". Martín-Cruces, José, García-Mesa, Yolanda, Garrido, Andrea, Muriel, Juan D.l, Cobo, Juan L., Maranillo, Eva, Vázquez, Teresa, Vega, José A. XXIX Congreso de la Sociedad Anatómica Española que tuvo lugar en la Universitat Jaume I y ha sido organizado por la Fundación Universitat Jaume I- Empresa en colaboración con la Sociedad Anatómica Española, celebrado los días 15, 16 y 17 de septiembre de 2021.

9. OTROS MÉRITOS O ACLARACIONES QUE SE DESEE HACER CONSTAR

- CURSOS:

1. Electrocardiografía para enfermeras. Conocimientos teóricos. Módulo 1 - edición 2. (9 CFC; 07-AFOC- 02136.1/2016).
2. Electrocardiografía para enfermeras. Conocimientos teóricos. Módulo 2 - edición 2. (9 CFC; 07-AFOC- 02136.1/2016).

3. Intervención nutricional en las situaciones clínicas más comunes en el anciano. (3,9 CFC; 09-14572; 19/10/2016).
4. Programa énfasis en enfermería oncológica (4,4 CFC; 09-016627-IN; 21/10/2016).
5. Programa énfasis en enfermería oncológica. Módulos 4 y 5. (09-015492-in; 21/10/2016).
6. Aspectos nutricionales en el medio extrahospitalario. Módulos 1-4. (6,9 CFC; 09-15681-MD; 19/10/2016).
7. Programa de formación continuada en nutrición y diabetes para enfermería (10,2 CFC; 07-AFOC-05105.0/2015; 23/09/2016).
8. 8. Programa de formación continuada en nutrición infantil para enfermería (4,9 CFC; 07-AFOC-05100.4/2015; 24/09/2016).
9. Bases de la enfermería de urgencias y emergencias (80horas; SATSE; 2016).
10. Dieta en el síndrome diarreico, estreñimiento y en la intolerancia a la lactosa (26/09/2016).
11. Los grupos de alimentos y la mujer embarazada (26/09/2016).
12. Alimentación del recién nacido, preescolar, escolar y en la adolescencia (26/09/2016).
13. Alimentación en la menopausia y en la vejez (26/09/2016).
14. Dieta hiperuricemia, la obesidad y en las ostomías (26/09/2016).
15. Dieta en la diabetes mellitus, en la diabetes gestacional y dieta en las hiperdislipemias (26/09/2016).
16. Dieta en la hipertensión arterial y en pacientes con VIH (26/09/2016).
17. Dieta preventiva del cáncer, dieta en el paciente oncológico y dieta en la enfermedad celíaca (26/09/2016).
18. Abordaje integral en el paciente con disfagia: una visión multidisciplinar (11/06/2017).
19. La preinscripción de ejercicio físico en el hipertenso. Normas generales de preinscripción de ejercicio físico (11/06/2017).

20. Aspectos nutricionales en el medio extrahospitalario ¿Realmente sabemos cuidar de nuestros mayores? Módulos I-IV. (11/06/2017)
21. Aspectos nutricionales en el medio extrahospitalario ¿Realmente sabemos cuidar de nuestros mayores? Módulo V. Nutrición enteral desde el ingreso hasta el domicilio. (11/06/2017).
22. Aspectos nutricionales en el medio extrahospitalario ¿Realmente sabemos cuidar de nuestros mayores? Módulo VI. Cuidados de enfermería en el paciente geriátrico. (11/06/2017).
23. Herramientas para la nutrición basada en la evidencia. (11/06/2017).

- PARTICIPACIÓN NOCHE JÓVENES INVESTIGADORES: 2019.
- PERTENENCIA GRUPO DE INVESTIGACIÓN ACREDITADO SINPOS (SISTEMA NERVIOSO PERIFÉRICO Y ÓRGANOS DE LOS SENTIDOS) DEL CLÚSTER DE BIOMEDICINA, CAMPUS DE EXCELENCIA INTERNACIONAL, UNIVERSIDAD DE OVIEDO: 2019-ACTUALIDAD.
- PROFESORA COLABORADORA DE HONOR (ANATOMÍA EN PEDAGOGÍA): CURSOS 2017-2018; 2018-2019.
- PROFESORA P.O.D. GRADO DE ENFERMERÍA, PRIMER CURSO (ASIGNATURA ANATOMÍA HUMANA): 180 HORAS (CURSOS ACADÉMICOS: 2019-2020; 2020-2021; 2021-2022) POR CONTRATO PREDOCTORAL SEVERO-OCHOA.

Anexo 2 – Artículos relacionados con la tesis

Feito J, Cebrián-Muiños C, Alonso-Morrondo EJ, García-Mesa Y, García-Piqueras J, Cobo R, García-Suárez O, Vega JA. Hyperplastic sensory corpuscles in nevus sebaceus of labia minora pudendi. A case report. J Cutan Pathol. 2018; 45:777-781.

Feito J, García-Suárez O, García-Piqueras J, García-Mesa Y, Pérez-Sánchez A, Suazo I, Cabo R, Suárez-Quintanilla J, Cobo J, Vega JA. The development of human digital Meissner's and Pacinian corpuscles. Ann Anat. 2018; 219: 8-24.

García-Piqueras J, García-Mesa Y, Feito J, García B, Quiros LM, Martín-Biedma B, Cobo T, Vega JA, García-Suárez O. Class I and Class II small leucine-rich proteoglycans in human cutaneous pacinian corpuscles. Ann Anat. 2019; 224: 62-72.

García-Piqueras J, García-Mesa Y, Cárcaba L, Feito J, Torres-Parejo I, Martín-Biedma B, Cobo J, García-Suárez O, Vega JA. Ageing of the somatosensory system at the periphery: age-related changes in cutaneous mechanoreceptors. J Anat. 2019 Jun; 234: 839-852.

García-Piqueras J, García-Suárez O, García-Mesa Y, García-Fernandez B, Quirós LM, Cobo R, Martín-Biedma B, Feito J, Vega JA. Heparan sulfate in human cutaneous Meissner's and Pacinian corpuscles. Anat Rec (Hoboken). 2020; 303: 2262-2273.

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García-Piqueras J, Cobo R, Cárcaba L, García-Mesa Y, Feito J, Cobo J, García-Suárez O, Vega JA. The capsule of human Meissner corpuscles: immunohistochemical evidence. J Anat. 2020; 236: 854-861.

Gutiérrez-Villanueva M, García-Mesa Y, García-Piqueras J, Cobo R, García-Suárez O, Vega JA, Feito J. The sensory innervation of the human nipple. Ann Anat. 2020; 229:151-156.

Cobo R, García-Piqueras J, García-Mesa Y, Feito J, García-Suárez O, Vega JA. Peripheral Mechanobiology of Touch-Studies on Vertebrate Cutaneous Sensory Corpuscles. Int J Mol Sci. 2020; 21: 6221.

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García-Suárez O, García-Mesa Y, García-Piqueras J, Salvo G, Cobo JL, Alba E, Cobo R, Feito J, Vega JA. The Cutaneous Biopsy for the Diagnosis of Peripheral Neuropathies: Meissner's Corpuscles and Merkel's Cells. The Cutaneous Biopsy for the Diagnosis of Peripheral Neuropathies: Meissner's Corpuscles and Merkel's Cells. In: Demystifying Polyneuropathy. Recent Advances and New Directions. P. Bozzetto Ambrosi Ed. IntechOpen, DOI: 10.5772/intechopen.73946. ISBN: 978-1-83881-192-1. Print ISBN: 978-1-83881-191-4. eBook (PDF) ISBN: 978-1-83881-193-8