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Distribution of TRPC1, TRPC3, and TRPC6 in the human thyroid

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ABSTRACT

Background: Little is known about the protein expression of the transient receptor potential canonical (TRPC) channels 1, 3, and 6 in the thyroid. Research in human tissue is insufficient. Our aim was to investigate the distribution of TRPC1, 3, and 6 in the healthy human thyroid.
Methods: Healthy samples were collected from seven nitrite pickling salt-ethanol-polyethylene glycol-fixed cadavers and from one patient who had undergone neck surgery (5 males, 3 females; median = 81.0, interquartile range = 6.5 years). The protein expression profiles of TRPC1, 3, and 6 were assessed using immunohistochemistry with knockout-validated antibodies. A monoclonal calcitonin antibody was used to detect calcitonin-producing C-cells.
Results: All samples were labeled as healthy, displaying age-appropriate signs of degeneration. TRPC1, 3, and 6 immunolabeling in thyrocytes showed irregular staining patterns leaving selected cells with intense staining, some without. The comparison of calcitonin- and TRPC1-, 3-, and 6-immunolabeled slides strongly suggested TRPC1, 3, and 6 expression in C-cells. Connective tissue showed no immunoreactivity.
Conclusions: This is, to the authors' knowledge, the first detailed description of the distribution of these channels in the human thyroid. We conclude that TRPC1, 3, and 6 are expressed in thyrocytes and C-cells of the human thyroid. Further studies are necessary to confirm these small-case-number results and to explore the relevance of these versatile channels in thyroidal health and disease.

1. Introduction

The family of transient receptor potential (TRP) channels constitutes a group of cellular, membrane-bound, non-selective ion channels, distributed across various tissues and cell types in both animal and human organisms [1]. These channels play a pivotal role in physiological processes, acting as essential mediators of sensory signals, signaling pathways, and contributing to various pathophysiological conditions. Notably, they are implicated in the pathogenesis of diseases affecting the cardiovascular, skeletal, renal, and nervous systems [2–4]. Currently, the mammalian TRP-channel family is subclassified into the canonical (TRPC), the melastatin (TRPM), the vanilloid (TRPV), the mucolipin (TRPML), the polycystin (TRPP), the ankyrin (TRPA), and the "no mechanoreceptor potential C" (TRPN) subfamilies [5,6]. The TRPC subfamily comprises seven members (TRPC1–7) of non-selective vastly Ca^{2+}-permeable cation channels [5]. They display a tetrameric structure, in which each monomer or subunit is composed of six transmembrane segments (S1–S6) with variably assembled cytoplasmic domains at each terminus. The S5 and S6 transmembrane segments of each monomer form the Ca^{2+}-permeable cation-pore [2], emphasizing the crucial role of TRPC channels in cellular Ca^{2+}-household and -signaling. They are manifoldly expressed in human organs, influencing a multitude of cellular processes such as transcription factor activation, apoptosis, and cell proliferation [7–10]. TRPC channels can function as receptor-operated Ca^{2+}-entry (ROCE) and/or store-operated Ca^{2+}-entry (SOCE) channels. Activation of the G protein-coupled receptor G_q, promotes phospholipase C (PLC)-mediated release of inositol-triphosphate (IP_3) and diacylglycerol (DAG). DAG can initiate ROCE by stimulating TRPC channels resulting in an increase in cytosolic Ca^{2+}-concentration. IP_3 in turn rather triggers the SOCE pathway by releasing Ca^{2+} from

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intracellular stores, such as the endoplasmic reticulum, ultimately activating store-operated channels (SOCs) in the plasma membrane resulting in Ca^{2+}-influx reinforcement [11,12]

Ca^{2+}-signaling also plays an essential role in the thyroid. In addition to the classical thyroid stimulating hormone (TSH)-evoked cyclic adenosine monophosphate (cAMP) pathway, TSH induces Ca^{2+}-signaling through PLC-IP_3 pathways [13]. Of note, also thyroidal cell proliferation and desoxyribonucleic acid (DNA)-synthesis are dependent upon Ca^{2+}-signaling [14,15]. Furthermore, regulation of TSH-receptor and thyroglobulin expression and synthesis are intricately linked to changes in intracellular Ca^{2+}-levels [16,17]. Previous studies have detected ribonucleic acid (RNA) sequences encoding selected TRPC channels in the human thyroid, and provided evidence suggesting their involvement in thyroidal Ca^{2+}-signaling pathways [18,19]. However, a comprehensive histological description of the respective protein expression and distribution is missing. With this in mind, a short summary of the physiology and anatomy of the thyroid is necessary.

Situated in the lower neck, the thyroid is an endocrine gland comprising two lobes connected by an isthmus [20]. It is located anterior to the trachea and inferior to the cricoid cartilage [21] (Fig. 1). The thyroid is critical in following functions [22]. First, the hormone production (triiodothyronine, T3; and tetraiodothyronine, T4) by the thyroid follicular epithelial cells (i.e., thyrocytes), that constitute a major portion of the thyroid tissue. These hormones act on various organs including the heart, lungs, and skeletal muscle [23]. Second, the secretion of the peptide hormone calcitonin by so-called C-cells as a response to increased serum Ca^{2+}-concentration [24]. Aforementioned thyrocytes form a single-layered epithelial layer lining the follicullar colloid in which T3 and T4 are stored (Fig. 1). In contrast, the C-cells, also known as parafollicular cells, constitute a minority. These cells are isolated or grouped within a common basal lamina with the follicular epithelial cells even though without connection to the follicular colloid (Fig. 1). [24]

Asghar and colleagues investigated expression of TRPC RNA in healthy and diseased thyroids using polymerase chain reaction (PCR). Their results suggest a significant involvement of Ca^{2+}-signaling including TRPC channel activity in the progression of thyroid cancer [13,18]. However, the authors had a focus on TRPC1 [18], ultimately disregarding players like TRPC3 and TRPC6 which are nevertheless also attributed a number of roles in various pathophysiological processes [3,4,26]. Moreover, TRPC1 is suggested to form complexes with TRPC3 [27], which in turn is known to build heteromers with TRPC6 [28,29]. This is critical since heteromerization substantially enlarges the spectrum of functions that TRPC tetramers can obtain – in health and disease [30]. In summary, there is need for a more detailed exploration of TRPC channel protein expression in the human thyroid to support further research on potential pharmacological targets [25]. Therefore, the aim of this study was to comprehensively investigate the TRPC1, 3, and 6 protein expression and distribution in the healthy human thyroid.

2. Methods and materials

2.1. Specimens

Thyroid samples were obtained from human adults who decided to donate their bodies to education and science after death and from one who had undergone neck surgery (Table 1). Five of the human tissue samples were obtained from male specimens, three from female. Median age at death or surgery was 81.0 with an interquartile range of 6.5 years.

Body donors had been fixed within 72 hours postmortem by retrograde perfusion with nitrite pickling salt-ethanol polyethylene glycol fixation (NEP) through the femoral artery according to Weigner's protocol [31]. Afterwards, immersion fixation was applied by placing the bodies in 3 % formalin and NEP-solution for up to 3 months until completion of the fixation process. Thyroid samples were obtained from the right and left lobe using punch biopsies during dissection courses at the anatomical institute.

The surgery sample had been obtained from the right lobe in the context of parathyroid adenoma surgery. Fixation was performed within the surgical schedule using 4 % phosphate-buffered formalin.

The fixated tissue samples were then embedded in paraffin as described before [32]. From that, 4 μm thick sections were prepared using a microtome and drawn onto microscopic slides.

Table 1
Overview of the samples (age [at death or surgery], sex, diagnosis/cause of death, tissue origin).

Number	Age	Sex	Diagnosis/Cause of death	Tissue Origin
1	81	Male	Parathyroid adenoma	Surgery
2	76	Female	Embolism	Body donor
3	75	Male	Septic shock, pneumonia	Body donor
4	82	Male	Kidney failure, metastatic prostate cancer	Body donor
5	83	Female	Cardiogenic shock	Body donor
6	81	Female	Cardiopulmonary insufficiency	Body donor
7	90	Male	Cardioembolic event	Body donor
8	81	Male	Multiple organ failure	Body donor

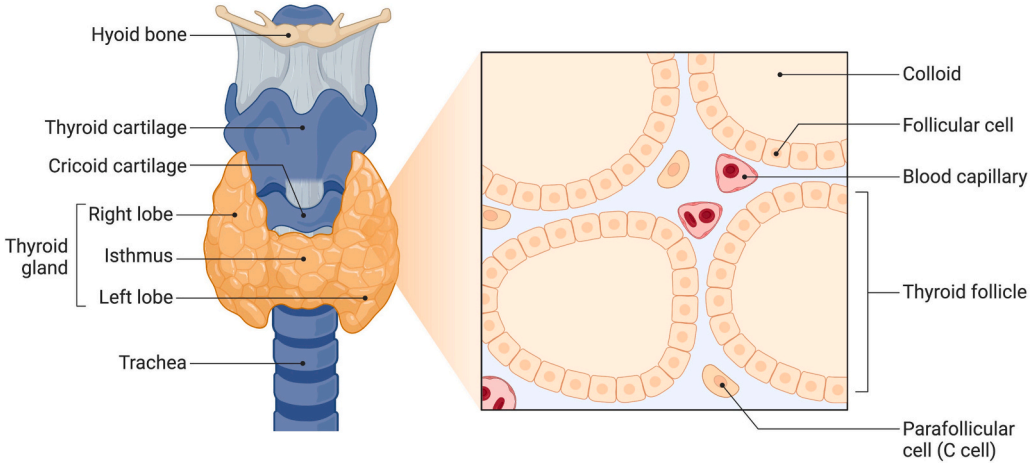

Fig. 1. Anatomy and histology of the thyroid. Follicular cells (i.e., thyrocytes) make the largest part of the tissue lining the thyroidal follicle. Calcitonin-producing parafollicular or C-cells are isolated or grouped around the follicles. Follicles are surrounded by tissue and close-meshed capillary network. Created with BioRender (Agreement number: WU267U400D).

All investigations were performed anonymously and in accordance with the guidelines of the Declaration of Helsinki. This study was approved by the Ethics Committee of the Saarland Medical Association (vote numbers: 163/20, and 130/21). Informed consent was obtained from the patient and the body donors.

2.2. Hematoxylin & eosin staining

Hematoxylin & eosin (H&E) staining was performed using standardized techniques [33]. Briefly summarized, samples were deparaffinized in 100 % Xylol for 15 min and rehydrated in decreasing concentrated ethanol solutions (2 × 100 %, 1 × 90 %, and 1 × 80 %, 5 min each). After 8-min-long staining in Ehrlich hematoxylin (C. Roth, Karlsruhe, Germany), the samples were washed in distillated water and blued in fluent tap water for 12 min. Staining in 0.1 % eosin followed (Central Chemical Storage, Saarland University, Saarbrücken, Germany). Finally, the samples were dehydrated using increasing ethanol concentration series (1 × 90 % and 2 × 100 % ethanol, and 3 × 100 % Xylol, 5 min each).

2.3. Immunohistochemistry

The polyclonal knockout-validated rabbit TRPC1 (ACC-010, Alomone Labs, Jerusalem, Israel), TRPC3 (ACC-016, Alomone Labs, Jerusalem, Israel), and TRPC6 antibodies (ACC-017, Alomone Labs, Jerusalem, Israel) were used.

Samples were incubated in citrate buffer in a heating incubator at 95°C for 60 min. Then, the primary antibody which was diluted 1:50 in phosphate-buffered saline was applied. For negative controls, a 1:500 diluted rabbit serum was used. After overnight incubation, a secondary antibody (horseradish peroxidase, goat anti-rabbit, A10547, Invitrogen AG, Carlsbad, CA, USA) and a chromogen (diaminobenzidine tetrachloride [DAB], SK-4103, Vector Laboratories, Burlingame, CA, USA) were applied. DAB incubation time was set at 5 min. Nuclear counterstaining with Ehrlich hematoxylin followed (C. Roth, Karlsruhe, Germany).

According to the manufacturer's information, the TRPC1, 3, and 6 antibodies are knockout-validated and generated to recognize the corresponding channels in mouse, rat, and human tissues [34–37]. In detail, the TRPC1 antibody detects the amino acid residues 557–571 of human TRPC1 (Peptide QLYDKGYTSKEQKDC), the TRPC3 antibody the residues 822–835 of murine TRPC3 (Peptide HKLSEKLNPSVLRC), and the TRPC6 antibody the residues 24–38 of murine TRPC6 (Peptide [C] RRNESQDYLLMDELG).

Additionally, immunohistochemistry was performed using a monoclonal rabbit calcitonin antibody generated to recognize the human calcitonin 1–32 peptide (SP17, Invitrogen, Thermo Fisher Scientific, MA, USA). Immunohistochemistry was performed as aforementioned for TRPC1, 3, and 6 except for a longer DAB incubation time of 12 min.

2.3.1. Histological identification of C-cells

Distinguishing C-cells from thyrocytes can be challenging [38]. The designation "C-cells" is derived from the term "clear cells," which refers to their low affinity for certain histological staining methods. Thyrocytes line the thyroidal follicle. Their size depends on their activity fluctuating from flat or inactive over cuboidal to columnar or highly active [39]. In an H&E stain normal C-cells cannot be delineated with complete certainty. Depending on cutting direction, they can appear like thyrocytes. C-cells can be round, polygonal or spindle shaped with a larger and lightened nucleus, in comparison to thyrocytes. The name "parafollicular cells" was introduced 1932 and widely used since, which can be misleading considering that less than 1 % of human C-cells are parafollicular [38]. As aforementioned, they often occur in the cellular lining of the follicles, though remaining separated by thyrocytes from the follicular content also called colloid [39].

2.4. Visualization

Histological slides were digitalized using the Nano Zoomer S210 (Hamamatsu, Japan). Microphotographs were taken using the image viewing software NDP.view2 from Hamamatsu (U12388–01, Hamamatsu, Japan). Representative microphotographs for the cohort were mostly obtained from the surgical specimen to enhance quality of illustration.

3. Results

3.1. Overview

All samples were tumor free and age-appropriate, as evaluated and labeled by trained pathologists. All cadaveric samples showed varying degree of autolysis but mostly intact tissue. The following description refers to all samples, unless differently mentioned.

H&E staining displayed a physiological lobar parenchymatous architecture and subarchitecture (Fig. 2). Thyrocytes and C-cells were identified according to the aforementioned criteria (Fig. 2C and D).

Immunohistochemical calcitonin staining displayed a sparse pattern of isolated and grouped calcitonin-produding C-cells (Fig. 3A and B). Follicular epithelial cells and connective tissue were immunolabeling-deficient. Negative control of the calcitonin antibody displayed no DAB-specific coloration (Fig. 3C).

3.1.1. TRPC1

TRPC1 immunoreactivity was detected in thyrocytes (Fig. 4). Throughout all samples, thyrocyte staining was heterogenous with respect to coloration intensity (Fig. 4). Few thyrocytes displayed no staining at all (Fig. 4B). C-cells displayed a homogenous immunoreactivity consistent with TRPC1 protein expression (Fig. 4C). In contrast, connective tissue was not immunolabeled. Negative control of the TRPC1 antibody displayed no DAB-specific coloration (Fig. 3D).

3.1.2. TRPC3

TRPC3 immunoreactivity was detected in thyrocytes (Fig. 5). Throughout all samples, thyrocyte staining was heterogenous with respect to coloration intensity (Fig. 5). Few thyrocytes displayed no staining at all (Fig. 5B). C-cells displayed a homogenous immunoreactive signal consistent with TRPC3 protein expression (Fig. 5C). In contrast, connective tissue was not immunolabeled. Negative control of the TRPC3 antibody displayed no DAB-specific coloration (Fig. 3E).

3.1.3. TRPC6

TRPC6 immunoreactivity was detected in thyrocytes (Fig. 6). Throughout all samples, thyrocyte staining was heterogenous with respect to coloration intensity (Fig. 6). Few thyrocytes displayed no staining at all (Fig. 6B and C). C-cells displayed a homogenous immunoreactive signal consistent with TRPC6 protein expression (Fig. 6C and D). In contrast, connective tissue was not immunolabeled. Negative control of the TRPC6 antibody displayed no DAB-specific coloration (Fig. 3F).

4. Discussion

4.1. Overview

The aim of this study was to investigate protein expression and distribution of TRPC1, 3, and 6 in the healthy human thyroid. All analyzed tissue samples exhibited extensive TRPC1, 3, and 6 protein expression. Immunohistochemistry revealed heterogenous staining of thyrocytes that was comparable in all specimens. Protein expression was also detected in C-cells.

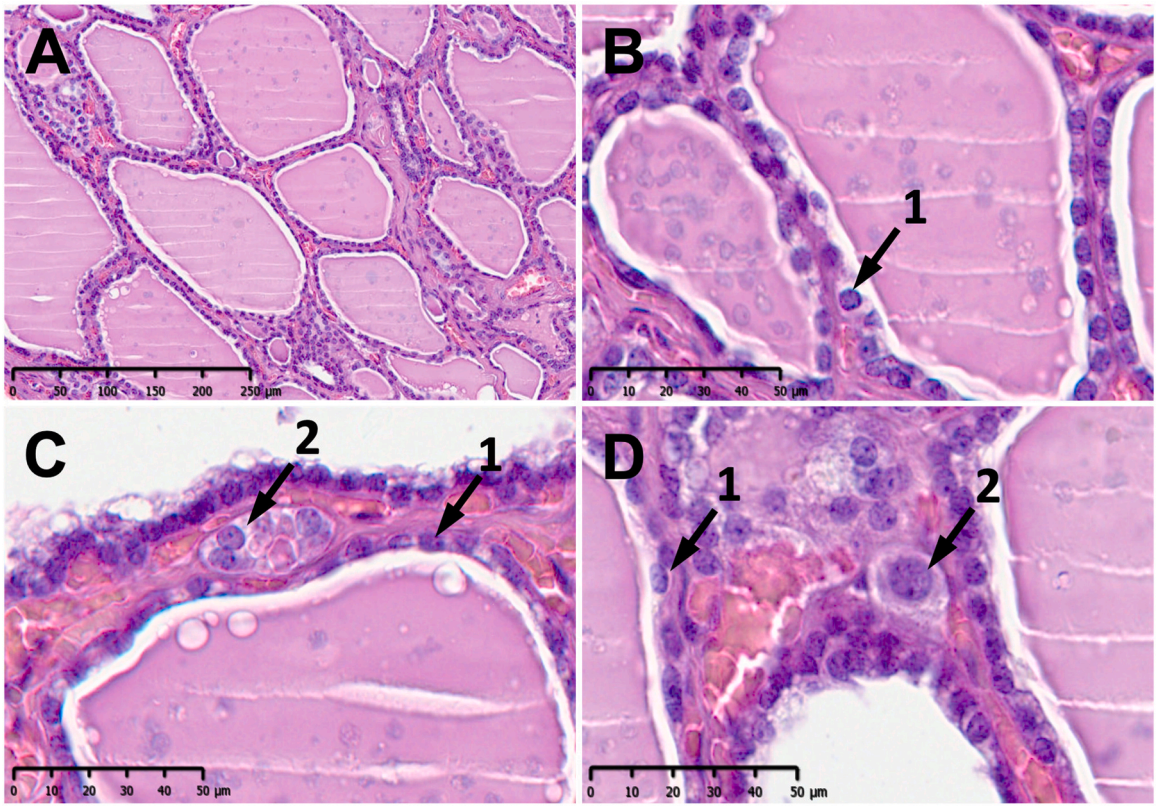

Fig. 2. Hematoxylin & eosin staining of the thyroid. Numerous colloid structures enclosed by thyrocytes and thyroid stroma (A; 10 × software magnification). Thyrocytes in higher magnification (B, C, and D; 40 × magnification). Grouped and isolated C-cells (C and D; 40 × magnification). Thyrocytes (1), C-cells (2).

4.2. TRPC1, 3, and 6 in thyrocytes

Whereas the relevance of TRPC1, 3, and 6 in thyroidal physiology and pathophysiology is not yet elucidated, the importance of Ca^{2+}-signaling is accepted [13,14,16,17]. TRPC1, 3, and 6 are integral in Ca^{2+}-signaling, which governs essential cancer-related processes such as migration, invasion, and proliferation [13,25]. The RNA of TRPC1, 3, and 6 is expressed in normal thyroid tissue [18]. The ability of these channels to regulate intracellular Ca^{2+}-levels makes them promising candidates for targeted therapies aimed at controlling thyroid cancer progression. Concretely, Asghar *et al.* demonstrated a significant role for TRPC1 in invasion, migration, and proliferation in human thyroid cancer cells [18]. Nevertheless, TRPC3 and 6 are important modulators of these cellular activities [25]. For instance, TRPC6 is known to play a central role in migration and invasion [40–43], whereas TRPC3 has been shown to influence cancer cells in several tissues through its role in calcium signaling and its interactions with other signaling pathways [44,45].

In experiments with rat thyroid cells exclusively expressing the TRPC2, a crucial role of this channel in various physiological processes was observed [15,46,47]. However, the TRPC2 protein is not expressed in humans, *Trpc2* being a pseudogene [48]. Still, to the authors' knowledge, a histological analysis of the expression of the remaining TRPC channels in human thyroid tissue has not been part of previous investigations. Asghar *et al.* presented in their publication a characterization of TRPC channels using molecular genetic methods [18]. Though it must be considered, as also mentioned by themselves, that the tissue might contain other cells expressing TRPC channels apart of thyrocytes [18]. Also, the detection of nucleic acids doesn't necessarily signify corresponding protein expression [49,50]. With our investigations we provide clear evidence that thyrocytes express the TRPC1, 3, and 6 proteins. Notably, TRPC channels have been implicated in other endocrine organs [51–53]. For instance, we recently provided evidence showing TRPC3 and TRPC6 protein expression in the human pancreas and parathyroid glands [54,55]. Summarizing, for the first time the TRPC1, 3, and 6 protein expression in human thyroidal tissue was evidenced. Further translational research is warranted to study the involvement of these channels in the cellular function of healthy and diseased thyrocytes, which is yet unclear.

4.3. TRPC1, 3, and 6 in C-cells

In contrast to thyrocytes, there are no studies published describing the expression of TRPC channels in C-cells to the authors' knowledge. This study provides evidence suggesting the protein expression of TRPC1, 3, and 6 in C-cells. As aforementioned identification of C-cells in light microscopy can be challenging due to their rarity [38] and uneven distribution [56]. To confirm our morphological C-cell identification, we performed calcitonin immunolabeling. Here, several stained cells, i.e., calcitonin producing C-cells, were detected. Although sections were sequentially prepared, no subcellular match was found with the TRPC1, 3, and 6 staining. Nevertheless, the morphology of calcitonin-immunostained cells supports the validity of our C-cell identification based on aforementioned descriptions in the TRPC1, 3, and 6 immunolabeled slides. Since studies have shown that the calcitonin peptide content displayed a strong correlation with the morphology of calcitonin-producing cells in the normal thyroid [57], it can be assumed that the calcitonin immunoreactive cells likely were active C-cells.

However, it must be considered that our samples were from older patients and that the number of calcitonin immunoreactive cells in elderly can greatly vary [58]. Therefore, the presented results cannot be translated one-to-one to younger specimens.

Altogether, these results warrant further research with larger case numbers also involving younger individuals. Nevertheless our investigations strongly indicate TRPC1, 3, and 6 protein expression in C-

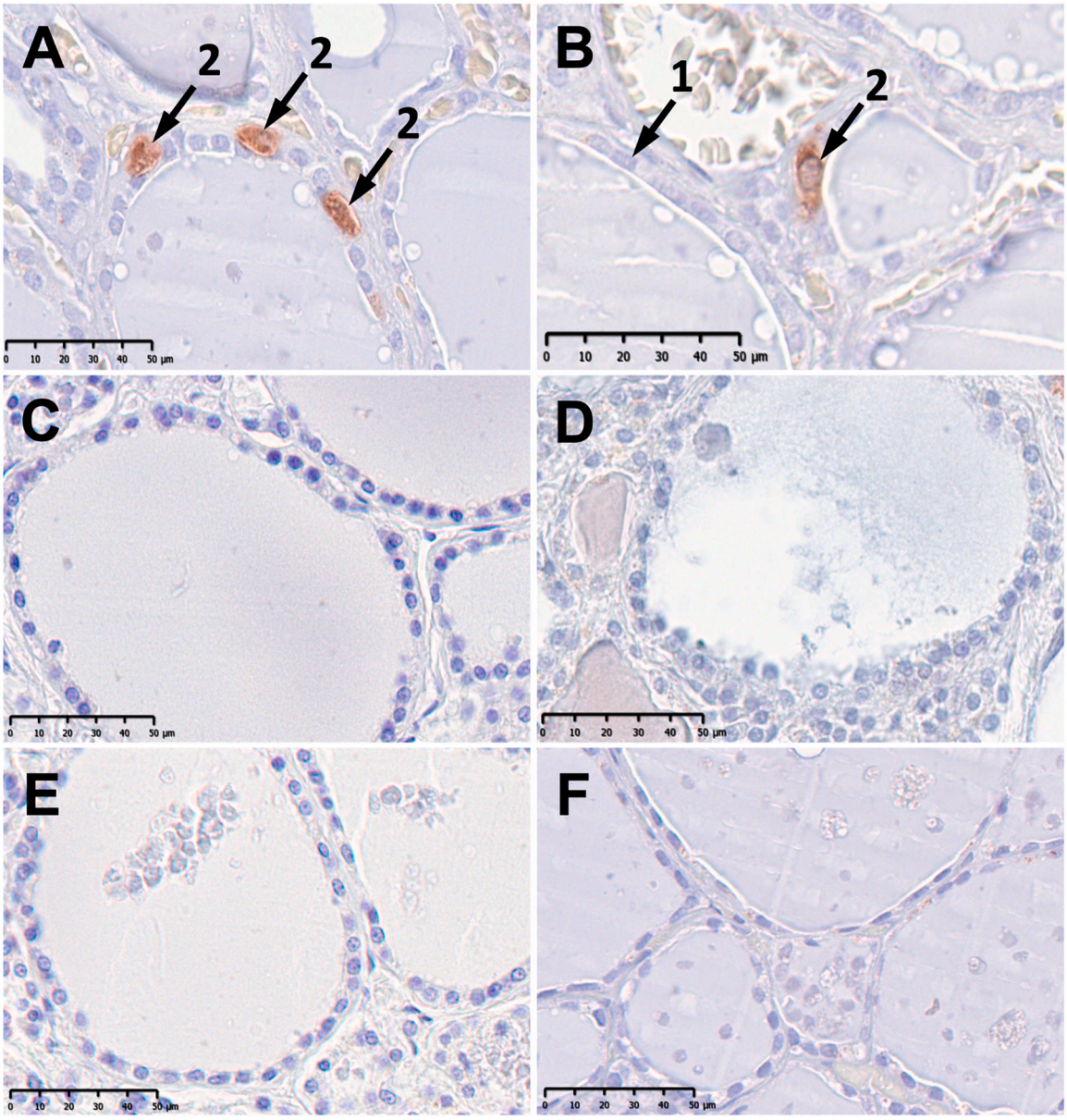

Fig. 3. Calcitonin expression in the human thyroid (A and B). Immunostaining with the calcitonin antibody in C-cells. Thyrocytes (1) and C-cells (2) are displayed (A and B; 40 × magnification). Negative control staining of the calcitonin (C; 40 × magnification), TRPC1 (D; 40 × magnification), TRPC3 (E; 40 × magnification), and TRPC6 (F; 40 × magnification) antibodies showed no DAB coloration.

cells. Considering that significant pathologies such as medullary thyroid carcinoma originate from C-cells [38] and that TRPC channels are involved in the development of cancer [25] further investigations could yield interesting results. In this context, targeted therapies hitting medullary thyroid carcinoma drivers such as the tyrosine kinase receptors VEGFR (vascular endothelial growth factor receptor), EGFR (epidermal growth factor receptor), and MET (mesenchymal-epithelial transition factor) yield promising results [59,60]. As a matter of fact, in the context of renal cell carcinoma a link between TRPC6 and MET has been observed as summarized in [4], foregrounding the increasing translational interest in studying TRPC channels and their potential involvement in Ca^{2+}-signaling-linked tumorigenesis and/or tumor progression.

4.4. Methodology and limitations

Immunohistochemistry is a highly specific method [61]. It can detect small amounts of protein. In connection with the histological evaluation, it not only allows for evaluation of corresponding protein expression but also protein distribution in a histological context. For this purpose, its superiority to molecular genetic methods is obvious [62]. A serious drawback is the lacking option of protein quantification. Furthermore, immunohistochemistry doesn't provide any information about the channel's integrity or activity. Besides, it must be addressed that the method of immunohistochemistry is a multi-stage process and variability can be introduced at any stage leading to a more difficult reproducibility of results. Tissue handling from body donors that were fixated postmortem can show, for example, protein degradation [63] or tissue liquification [64]. Importantly, the surgical sample showed similar results, ultimately indicating the transferability of the findings [65]. Furthermore, tissue processing can lead to loss of information of its natural state. While formalin as fixative method is widely used, cross-linking of proteins as significant characteristic can interfere with the antigens' ability to react with the primary antibody [66,67]. In the

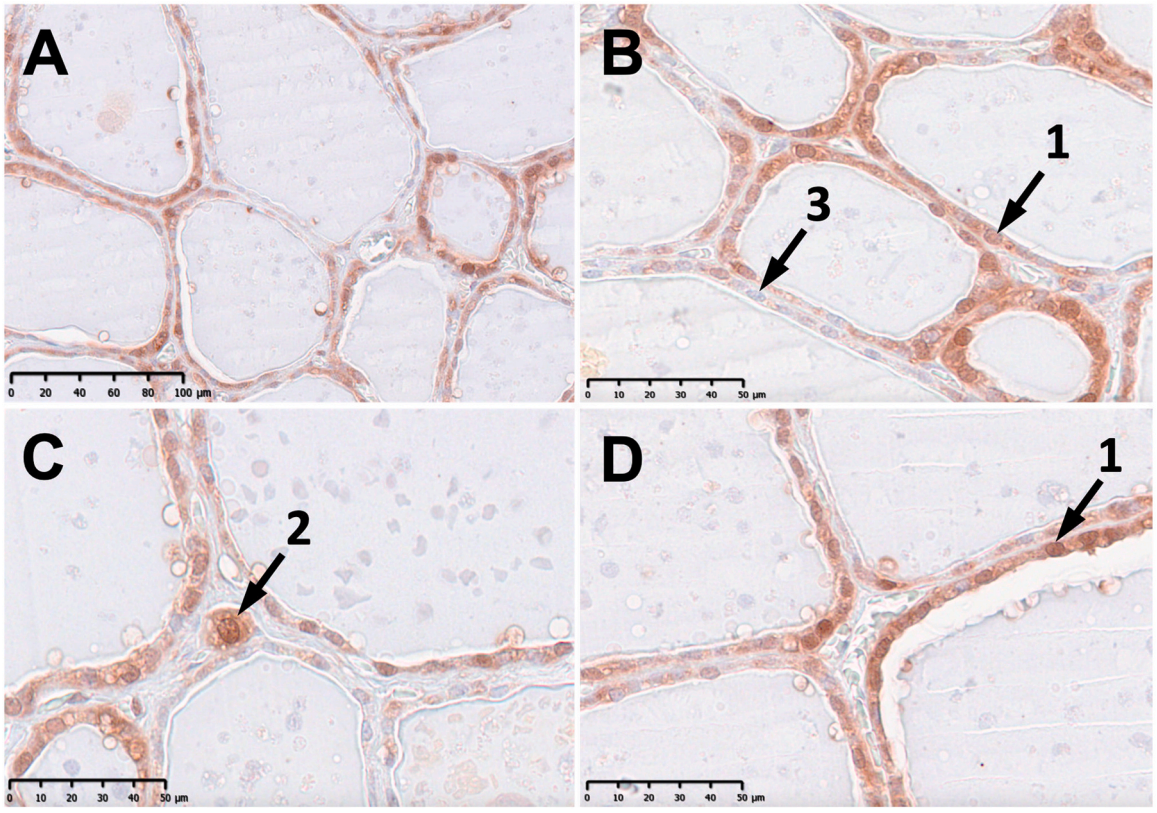

Fig. 4. TRPC1 protein expression in the human thyroid. Overview (A; 20 × magnification) and higher magnification microphotographs (B, C, and D; 40 × magnification). Immunostaining-positive thyrocytes (1), C-cells (2), and immunostaining-deficient thyrocytes (3) are displayed.

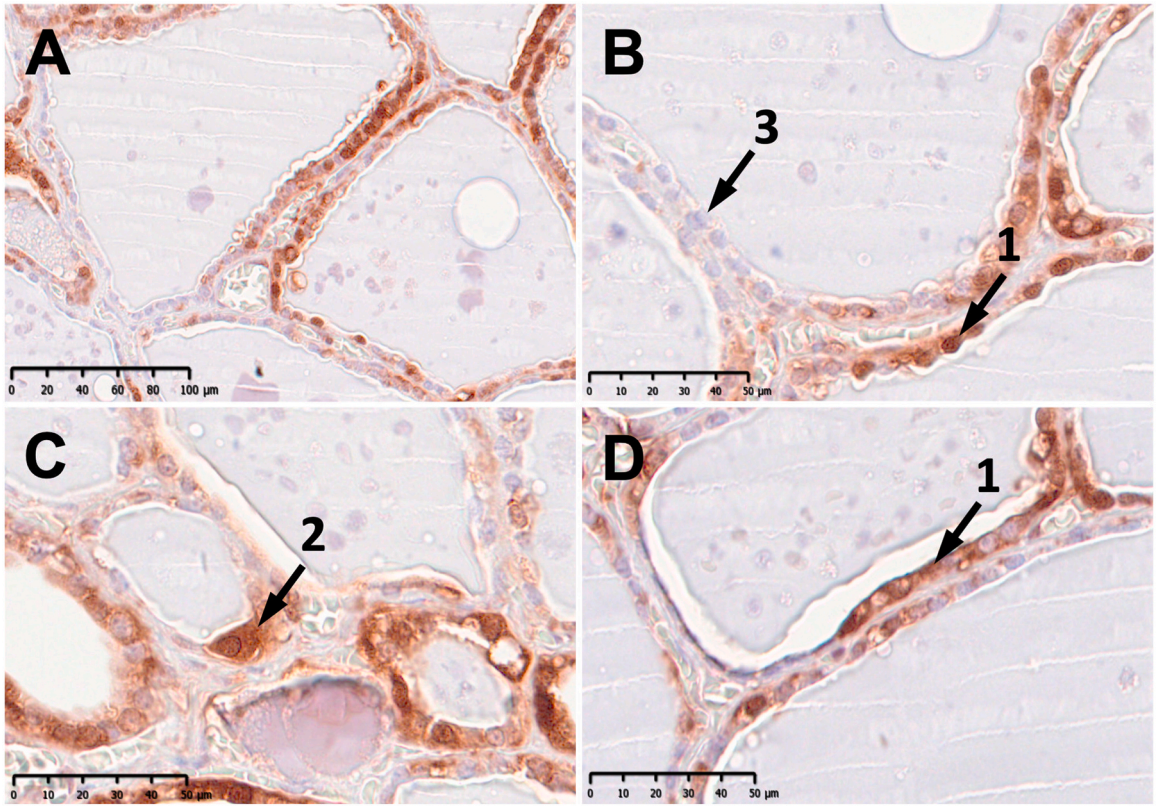

Fig. 5. TRPC3 protein expression in the human thyroid. Overview (A; 20 × magnification) and higher magnification microphotographs (B, C, and D; 40 × magnification). Immunostaining-positive thyrocytes (1), C-cells (2), and immunostaining-deficient thyrocytes (3) are displayed.

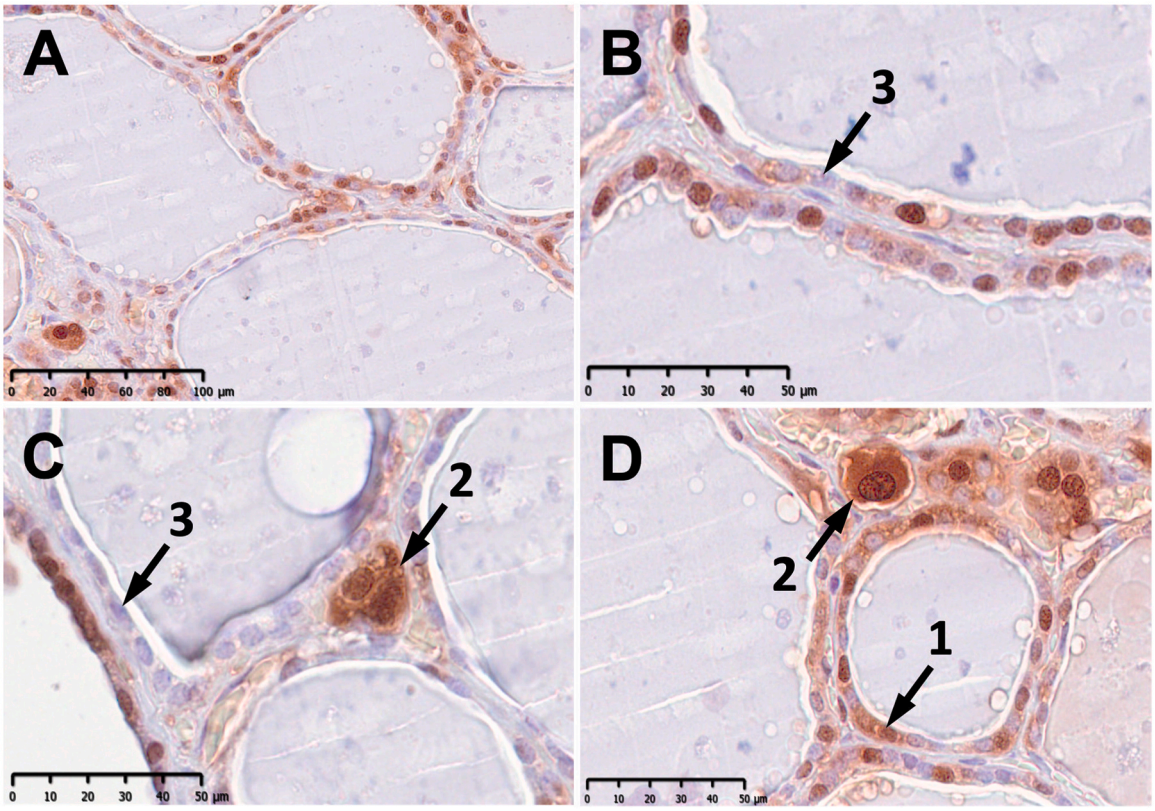

Fig. 6. TRPC6 protein expression in the human thyroid. Overview (A; 20 × magnification) and higher magnification microphotographs (B, C, and D; 40 × magnification). Immunostaining-positive thyrocytes (1), C-cells (2), and immunostaining-deficient thyrocytes (3) are displayed.

process of antigen retrieval, the conditions of heating and the pH of the buffer solution during the heating process can influence the effectiveness of the antigen retrieval [62]. Even though, our antigen retrieval was performed using standardized techniques, one cannot exclude the possibility of undetected antigens. Moreover, no studies showing knockout validation for the used monoclonal calcitonin antibody exist (SP17, Invitrogen, Thermo Fisher Scientific, MA, USA). Uncertainty with regard to potential lack in specificity is therefore given, although the staining pattern was very plausible to reflect the C-cell distribution.

The heterogenous staining pattern described in TRPC1, 3, and 6 immunohistochemistry could be due to the above-described limitations. With regard to the surgery sample, one cannot exclude the possibility that neighbored thyroid tissue could have been affected on a biochemical level from abnormal changes due to parathyroid adenoma, even though the risk remains small as illustrated by previous studies, that investigated TRPC channels using tumor nephrectomy tissue [36,68]. As aforementioned, form and structure of thyrocytes can vary depending on the state of activity. A correlation between thyrocyte activity and staining intensity is possible [69,70]. As a matter of fact, highly prismatic and columnar cellular shape frequently corresponded to increased staining intensity in all immunoreactive stainings with TRPC1, 3 and 6 antibodies, leaving this open for further interpretation. Finally, it also needs to be kept in mind that staining intensity doesn't necessarily correlate with protein expression level.

This study analyzed a small case number. Thus, inter- and intra-individual variances could have been missed. Additionally, all samples were obtained from older patients. The results described in this study do not mirror the conditions in younger specimens. Further investigations are necessary to determine the translationability of our results.

5. Conclusions

The TRPC1, 3 and 6 proteins are expressed in thyrocytes and C-cells of the human thyroid. Further studies are needed to investigate the physiological function of these channels in thyrocytes and C-cells, and to assess their involvement in cell-derived pathologies.

Funding

This study was not funded by any exterior sources.

Ethical statement

All investigations were implemented anonymously, approved by the Ethics Committee of the Saarland Medical Association (163/20, 130/21), and performed in accordance with the guidelines of the Declaration of Helsinki. Informed consent was obtained from the patient and the body donors.

CRediT authorship contribution statement

Mathias Wagner: Writing – review & editing, Resources. **Coline M. Diebolt:** Writing – review & editing, Methodology, Investigation. **Emilie Kirstein:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Jan M. Federspiel:** Writing – review & editing, Formal analysis. **Colya N. Englisch:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Thomas Tschernig:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Alessandro Bozzato:** Writing – review & editing, Resources. **Dirk Schaudien:** Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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