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Published in:
Neuroendocrinology

DOI:
[10.1159/000329044](https://doi.org/10.1159/000329044)

2011

[Link to publication](#)

Citation for published version (APA):

Landerholm, K., Falkmer, S., Jarhult, J., Sundler, F., & Wierup, N. (2011). Cocaine- and Amphetamine-Regulated Transcript in Neuroendocrine Tumors. *Neuroendocrinology*, 94(3), 228-236.
<https://doi.org/10.1159/000329044>

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5

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Cocaine- and Amphetamine-Regulated Transcript in Neuroendocrine Tumors

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Short title: CART in Neuroendocrine Tumors

Key words: Cocaine- and amphetamine-regulated transcript, Neuroendocrine tumors, Endocrine pancreatic tumors, Gastrointestinal tract, Medullary thyroid carcinomas, Regulatory peptides

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Abstract

Background/Aims: Cocaine- and amphetamine-regulated transcript (CART) is an anorexigenic regulatory peptide highly expressed in the brain's appetite control centers, but also in peripheral neurons and in endocrine cells in the adrenal medulla, thyroid, pancreatic islets, and in the gastrointestinal tract. Plasma levels of CART were recently shown to be elevated in patients with neuroendocrine tumors (NETs), but the cellular sources of CART in NETs have remained unknown. The aim of the study was to establish whether CART is expressed in various types of NETs and, if so, to examine the frequency, distribution and phenotype of CART-expressing cells.

Methods: Tumor specimens from 133 NETs originating in the stomach, ileum, rectum, pancreas and thyroid were examined with immunohistochemistry and *in situ* hybridization. The expression of CART was quantified and the CART-expressing cells were phenotyped by double staining for established markers and hormones.

Results: CART-expressing tumor cells were found in the majority of the examined NETs. The expression pattern of CART was highly heterogeneous not only between tumors, but also within individual tumors. In 14% of the NETs, CART was found in a major population of the tumor cells.

Conclusion: CART is produced in the majority of NETs, regardless of tumor origin. This likely explains the elevated levels of circulating CART in certain NETs patients, as recently described. CART could therefore prove to be a useful tool in the diagnostics of NETs not only in blood samples, but also in histopathological specimens.

Introduction

Cocaine- and amphetamine-regulated transcript (CART) is a multifunctional regulatory peptide produced by central and peripheral neurons [1, 2] and by neuroendocrine cells in, for example, the pancreatic islets [3–5], the mucosa of the gastrointestinal (GI) tract [6, 7], the thyroid [8], and the adrenal medulla [9]. Data obtained so far indicate that, at least in the gastro-entero-pancreatic (GEP) region, CART does not reside in an endocrine cell of its own, but is rather coexpressed with various other peptides or amines serving as principal hormones. In the GI tract of humans and rats, for instance, CART is expressed in the gastrin-producing G cells in the antral portion of the stomach, as well as in endocrine cells in the upper small intestine [6, 10]. Likewise, it has been known for decades that GEP neuroendocrine tumors (NETs) frequently are multihormonal, i.e. in a single tumor several biologically active peptides and amines may be expressed in addition to the one giving rise to the main symptoms [11–14]. For instance, ileal carcinoids arising from EC cells invariably produce the monoamine serotonin, but in addition often tachykinins, notably substance P and the related neuropeptide K (NPK) [12, 15].

Regarding the physiological functions of CART, recent studies have revealed that CART is upregulated in several types of endocrine islet cells during fetal and neonatal development as studied in mice and rats [4, 5]. Further, CART regulates insulin, glucagon, and somatostatin secretion, and is upregulated in beta cells in a number of rat models of type 2 diabetes [16]. A physiological role of CART in the regulation of islet hormone secretion gains further support from studies showing that both CART null mice, and humans carrying a mutated CART gene, develop obesity and signs of type 2 diabetes [17–19].

So far, very little is known about the expression of CART at the cellular level in NETs; at hand is only a brief notice on immunohistochemically identified CART in a well differentiated insulin-producing pancreatic tumor (“insulinoma”), as opposed to a less-differentiated one with no demonstrable CART [5]. We therefore thought it worthwhile to examine in more detail the frequency, distribution pattern, and when possible the phenotype of CART-expressing cells in NETs.

In the present study, specimens from a large number of NETs were examined for the expression of CART by immunohistochemistry (IHC) and, in some instances, also by *in situ* hybridization (ISH). The obtained results revealed a remarkably heterogeneous CART expression. Interestingly, in some NETs, CART was expressed in the majority of tumor cells.

Materials and Methods

Tissue material and routine histopathological examinations

Paraffin-embedded specimens from gastric (n = 28), ileal (n = 58) and rectal (n = 15) carcinoids, endocrine pancreatic tumors (EPTs) (n = 9) and medullary thyroid carcinomas (MTCs) (n = 23) were used. The pancreatic tumors consisted of three insulin-producing, one gastrin-producing, one pancreatic polypeptide- (PP) producing and four EPTs with no production of islet-specific hormones. The specimens represented both primary tumors and metastases. Before inclusion, sections from all specimens were reexamined with routine histopathological stainings and IHC for chromogranin A (CgA) and synaptophysin in order to verify the neuroendocrine origin. In order to calculate the Ki-67 proliferation index, all included NETs were examined with MIB-1 antiserum. This study was approved by the Regional ethical review board at Linköping University, Sweden.

Immunohistochemistry

Indirect immunofluorescence was used throughout. Briefly, sections (5 µm thickness) were cut and mounted on slides, deparaffinized and rehydrated. Prior to immunostaining, antigen retrieval was performed by boiling sections in 0.01M citrate buffer (pH 6.0) in a microwave oven for 2x7 min at 650 W. Sections were incubated with primary antibodies, diluted in PBS with 0.25% BSA and 0.25% Triton-X100, overnight at 4° C. After rinsing 2x10 min with PBS containing 0.25% Triton-X100, sections were incubated with secondary antibodies with specificity for rabbit, goat, sheep, guinea pig, or mouse IgG, and conjugated with Cy2, Texas Red, or AMCA (Jackson, West Grove, PA, USA) for 1 h at room temperature. Sections were again rinsed and then mounted in 1:1 PBS and glycerol. Double and triple immunostainings were performed in order to examine possible coexistence of CART and hormones or established tumor markers. For this purpose primary antibodies raised in

different species were used in combination with secondary antibodies carrying different fluorophores. All specimens were evaluated independently by at least two experienced pathologists.

Antibodies

Two different antisera against CART were used: goat polyclonal anti-CART (code N-20, dilution 1:800, Santa Cruz Biotechnology, Santa Cruz, CA, USA) [20] and rabbit polyclonal anti-CART (code 12/D, dilution 1:1,600, kindly provided by Prof. Michael J. Kuhar, Emory University, Atlanta, GA, USA) [4]. Importantly, the same staining pattern was seen with the two antibodies and with five additional CART antisera (kindly provided by Novo Nordisk, Målöv, Denmark). The specificity of all CART antibodies was verified by lack of staining in the GI tract of CART null mutant mice [17]. In addition, antibodies against calcitonin gene-related peptide (CGRP) (code M8513, dilution 1:640, Euro-Diagnostica, Malmö, Sweden) [4], CgA (code C-SP1, dilution 1:300, Inkstar, Stillwater, MN, USA) [21], gastrin (code 7835, dilution 1:1,280, EuroDiagnostica) [22], ghrelin (code 486-1, dilution 1:2,560, Phoenix, Belmont, CA, USA) [22], glucagon (code 8708, dilution 1:5,120, EuroDiagnostica) [4], insulin (code 9003, dilution 1:2,560, EuroDiagnostica) [4], NPK (code NPK4, dilution 1:600, kind gift from Prof. E. Theodorsson, Linköping University, Linköping, Sweden) [8], PP (code AHP 515, dilution 1:640, Serotec, Oxford, UK) [4], serotonin (code 20080, dilution 1:1,600, DiaSorin, Stillwater, NM, USA) [22] and the cell proliferation marker Ki-67 (code M7187, dilution 1:80, DAKO, Glostrup, Denmark) [4] were used. All antibodies have been used previously for IHC and tested for possible cross-reactivity with chemically related substances as well as for unwanted reactions in double and triple stainings.

***In situ* hybridization**

A synthetic oligodeoxyribonucleotide probe, complementary to the sequence 180–209 of CART mRNA (accession number: NM_004291), was used. The probe was 3'-endtailed with [³⁵S]-dATP (Perkin Elmer, Stockholm, Sweden). The ISH protocol has been described previously [23]. In brief, deparaffinized and rehydrated sections were treated with proteinase K (10 µg/ml, Sigma, St. Louis, MO, USA) for 30 min at 37° C, fixed in 4% paraformaldehyde for 15 min, washed for 5 min in PBS,

and then acetylated with 0.25% acetic anhydride in 0.1M triethanolamine for 10 min. Thereafter, sections were dehydrated in graded ethanols and air dried. Hybridization was carried out in sealed moisturizing chambers at 37° C overnight, using a probe concentration of approximately 1 pmol/ml, followed by stringent posthybridization washing (1x SSC; 0.15M NaCl, 0.015M sodium citrate). The slides were dipped in Ilford K.5 emulsion and stored in lightsealed boxes at 4° C for 21 days. They were then developed in Kodak D-19, fixed in Kodak Polymax and mounted in Kaiser's glycerol gelatine. Hybridization in the presence of 100-fold excess of unlabeled probe was used as negative control.

Imaging

Immunofluorescence was examined in an epi-fluorescence microscope (Olympus BX60). ISH was analyzed in bright-field. Images were taken with a digital camera (Nikon DS-2Mv).

Grading of CART-expression

When examining the specimens for CART immunoreactivity (IR) under the microscope in the same visual field (25x objective), a grading between 0 and 3 was introduced. Tumors with no CART-IR cells were designated 0; tumors with a few (1–10) CART-IR cells per visual field were designated 1; tumors with many (10–100) CART-IR cells per visual field were designated 2, and tumors with more than 100 CART-IR cells were designated 3, in most of them more than 50% of the cells were CART-IR.

Results

Routine histopathological examinations

The NET diagnosis and the tumor origin was confirmed in all 133 specimens. The median Ki-67 proliferation index was < 1% (range 0–10%) with the highest proliferation in the EPTs and the lowest in the rectal carcinoids. In accordance with the WHO 2010 classification [24], 116 tumors (87%) were graded as G1 with Ki-67 index \leq 2% and 17 tumors (13%) as G2 with Ki-67 index 3–20%. There were no G3 tumors.

Immunohistochemistry

Regardless of tumor origin, CART-IR was detected in tumor cells in the majority (80%) of all examined NETs, but the IR intensity varied markedly from cell to cell. There was no obvious difference in frequency of CART-IR cells between primary tumors and metastases. Frequently, the distribution of CART-IR cells was heterogeneous, with certain areas completely devoid of such cells, and other areas displaying dense accumulations. In NETs of the GI tract, the submucosal areas often had a higher density of CART-IR cells than deeper-lying areas. In many tumors, the CART-IR cells occurred as single cells, or as small cell clusters randomly dispersed in the tumor tissue. Interestingly, in a substantial proportion (9–33%) of NETs from all locations, CART-IR cells were the predominating tumor cells. The results are summarized in Table 1, and illustrated in Figure 1.

The clinical course could be evaluated in 12 of the 19 patients with the highest frequency of CART-IR cells (grade 3). Only in one case with a widely disseminated MTC was a clear weight loss noted.

In order to characterize the phenotype of the CART-IR cells, double and triple immunostainings were performed with antibodies to the main tumor hormones, to hormones causing clinical symptoms, or to established NET markers. In gastric carcinoid cells CART frequently coexisted with CgA, but never with ghrelin, which is a quite frequent constituent of such tumors. In ileal carcinoids CART invariably coexisted with CgA and serotonin (Fig. 2 A–C), and frequently also with the tachykinin NPK (Fig. 2 D–F). In rectal carcinoids, the CART coexistence pattern was more complex; many tumors contained a variety of hormonal peptides in separate subpopulations of cells, and CART was found to coexist with glucagon (Fig. 2 G–I), occasionally with somatostatin, but not with ghrelin, PP or serotonin. A similarly complex coexistence pattern was also evident in EPTs. For instance, the coexistence of CART and gastrin varied within the gastrin-producing EPT, and among the insulin-producing EPTs a clear pattern emerged in only one tumor, i.e. invariable coexistence with insulin (Fig. 2 J–L). In the MTCs, an overwhelming majority of CART-IR cells also contained CgA, calcitonin and CGRP (Fig. 2 M–O).

Further, to investigate whether CART expression could be associated with cell proliferation, double stainings for CART and Ki-67 were performed. As expected [25], and also confirmed already in the routine examinations at inclusion of the NETs, cells with Ki-67-IR nuclei were scarce, only a few were seen in each section. When comparing tumors or tumor areas rich in CART-IR cells with those devoid of CART, there was no visible difference in the frequency of Ki-67-IR cells. Indeed, we failed to detect any tumor cells coexpressing CART and Ki-67.

***In situ* hybridization**

In order to confirm that CART-IR represented CART mRNA expression, ISH was performed in 15 tumors (representing low to high CART expression by IHC) using previously established oligoprobes. In tumors or tumor areas with numerous CART-IR cells, a distinct and specific mRNA signal was also revealed (Fig. 3 A, C), whereas areas devoid of CART-IR cells were correspondingly lacking mRNA signal. Control sections hybridized in the presence of excess of unlabeled probe displayed only scattered background labeling (Fig. 3 B, D).

Discussion

Knowledge of CART in NETs is exceedingly meager. We have previously made a preliminary observation indicating the presence of CART in an insulin-producing EPT [5]. This observation became one impetus for Bech *et al.* [26] to perform a comprehensive investigation of circulating CART, which was found to be elevated in patients with a wide range of NETs. In the present study, histopathologically screened tissue material from a similarly wide range of NETs was analyzed in order to quantify and characterize CART-producing tumor cells. CART was found to be expressed in all the examined types of NETs, but with wide variations between NETs of the same origin, and also within individual tumors. This remarkable heterogeneity seems to set CART apart from many other tumor markers and messengers that usually display a more homogenous expression pattern. In a subset of NETs, regardless of tumor origin, a majority of the tumor cells expressed CART, indicating that NETs from all locations have the potential of producing CART also in large quantities.

This wide and heterogeneous expression of CART in NETs raises the question of a functional significance of CART in endocrine neoplasia. The present knowledge of peripheral CART effects in humans under normal conditions is limited, but it has been shown that humans with mutated CART become obese [19]. Similarly, CART affects islet hormone secretion in rodents [16] and CART-deficient mice have abnormal glucose regulation and gradually develop obesity [17]. A possible association between these anorexigenic properties of CART and cachexia in NET patients could therefore be plausible, but available data do not favor such a relationship in experimental EPT models [3]. Neither was such a relationship supported by the results in the present study. It should also be noted that any comparisons between the normal physiological and the pathophysiological function of CART in NETs need to be made with caution because of the uncertainty regarding the molecular identity of CART found in peripheral blood [26].

Bech *et al.* [26] reported that in some types of NETs, the levels of circulating CART were higher in patients with tumor progression than in those with stable disease. It is not established whether the raised CART level is the cause of or occur as a result of tumor progression. The relation between progression and CART expression was examined in the present study by means of double stainings for the cell proliferation marker Ki-67 and CART. The analyses did not identify any correlation between proliferation and CART expression. Also, in previous studies CART had no effect on the proliferation of clonal pancreatic beta cells [4].

The biosynthesis of CART mimics that of most other neurohormonal peptides in that CART is processed in the Golgi apparatus with the final tailoring taking place in the secretory granules, where the mature peptide is then stored [27]. It could therefore be hypothesized that the levels of circulating CART in the blood would be inversely related to the degree of differentiation of the tumor cells in NETs. Creutzfeldt *et al.* [28] noted more than three decades ago in insulin-producing EPTs that poorly differentiated tumor cells with an underdeveloped Golgi apparatus and few secretory granules were unable to convert proinsulin into insulin and also to store the hormone. Instead, proinsulin was instantly secreted into the blood resulting in drastically raised plasma hormonal levels, but only faint

IR in the tumor cells. The same mechanisms could possibly be applied to CART, but the uniform results of CART-IR intensity and CART mRNA ISH labeling density obtained in this study do not favor such an interpretation.

NETs are known to manufacture a plethora of regulatory peptides and non-peptide messengers, including amines [11–14, 29, 30]. It is not uncommon that such messengers coexist in one and the same tumor, and sometimes even in one and the same tumor cell. Nevertheless, one of the hormones, usually the one most abundantly expressed, predominantly gives rise to the clinical symptoms when such are evident. Other hypersecreted, coexisting messengers may add to, or occasionally mask, the symptoms. Also in normal tissues, CART is coexpressed with other peptides and amines in various neuroendocrine cells [4–6, 8, 16]. Double immunostainings were performed to delineate the coexpression patterns of CART in the various types of NETs. A consistent pattern was found in gastric and ileal carcinoids as well as in MTCs, whereas rectal carcinoids and EPTs displayed a more complex pattern. Possibly, these differences reflect the generally higher complexity of messenger coexpression in rectal carcinoids and EPTs.

In conclusion, this study shows that NET cells, regardless of origin, are capable of producing CART in varying, and sometimes large, amounts. Together with the previous data [26] on raised plasma levels of CART in NET patients, it is plausible that circulating CART is of tumor origin. A general neuroendocrine marker, in addition to presently used chromogranins, would be valuable in the clinical and histopathological diagnosis of NETs, as also argued by Bech *et al.* [26]. Their studies showed that CART could be a good candidate in clinical diagnostics. The findings of the present study suggest that CART is an interesting candidate for use as a histopathological diagnostic tool to distinguish NETs from other neoplasias. Whether CART in NETs is a friend or foe has to await further studies.

Acknowledgements

Linda Johansson, Jeanette Karlsmo, Britt-Marie Nilsson and Doris Persson are acknowledged for excellent technical assistance. This work was supported by the Swedish Research Council (Projects

No. 522-2008-4216, K2009-55X 21111-01-4, K2007-55X-04499-33-3), Faculty of Medicine at Lund University, the Royal Physiographic Society in Lund, Futurum – Academy for Healthcare at the Jönköping County Council, the Foundation for Clinical Cancer Research in Jönköping, the Novo Nordisk, Gyllenstiernska Krapperup, Fredrik and Ingrid Thuring, Magnus Bergwall, Albert Pålsson, and the Swedish Society of Medicine Foundations.

Disclosure Statement

None of the authors have anything to disclose.

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Table 1. Frequency of CART immunoreactive (IR) cells in neuroendocrine tumors (NETs)

NET type	Grade 0	Grade 1	Grade 2	Grade 3
Gastric carcinoid (n=28)	7 (25%)	12 (43%)	6 (21%)	3 (11%)
Ileal carcinoid (n=58)	11 (19%)	30 (52%)	11 (19%)	6 (10%)
Rectal carcinoid (n=15)	0	5 (33%)	5 (33%)	5 (33%)
EPT (n=9)	3 (33%)	3 (33%)	0	3 (33%)
MTC (n=23)	6 (26%)	12 (52%)	3 (13%)	2 (9%)
All locations (n=133)	27 (20%)	62 (47%)	25 (19%)	19 (14%)

Grade: 0 = No immunoreactive (IR) cells; 1 = 1-10 IR cells; 2 = 10-100 IR cells; 3 = >100 IR cells in the same visual field (25x).

EPT = endocrine pancreatic tumor; MTC = medullary thyroid carcinoma.

Figure legends

Fig. 1. CART-IR cells in NETs from stomach (**A, B**), ileum (**C, D**), rectum (**E, F**), and in an MTC (**G, H**). The tumors were selected to illustrate, at low (**A, C, E, G**) and high (**B, D, F, H**) magnification, the abundance of CART-expressing cells in certain NETs regardless of tumor origin. Scale bar in **A** = 100 μm (for left panels); in **B** = 50 μm (for right panels).

Fig. 2. Ileal carcinoid (**A–F**). Double immunostaining for CART (**A**), serotonin (**B**), and merged (**C**). All CART-expressing cells also contain serotonin, but many serotonin-containing cells lack CART. Although CART (**D**) coexists with the tachykinin NPK (**E**) in many cells, there are some NPK cells that lack CART (**F**, merged). Arrowheads indicate coexpression. Rectal carcinoid (**G–I**). Most of the CART-IR cells (**G**) are also glucagon-IR (**H**) (indicated by arrowheads). However, a few scattered CART-IR cells lack glucagon, and vice versa. Such cells thus remain red and green, respectively, in the merged image (**I**). Insulin-producing EPT (**J–L**). Most of the CART-IR cells (**J**) are identical with insulin-IR cells (**K**) (indicated by arrowheads), but a minority of the tumor cells contain either CART or insulin, since they remain red and green, respectively, in the merged image (**L**). CART-IR cells devoid of insulin indicated by arrows. MTC (**M–O**). The vast majority of CART-IR cells (**M**) also store CGRP (**N**) (exemplified by arrowheads). However, many of the CGRP cells lack CART (**O**, merged). Arrowheads indicate coexpression. Scale bar in **A** = 50 μm (for **A–I**), in **M** = 100 μm (for **J–O**).

Fig. 3. *In situ* hybridization micrographs with labeling for CART mRNA (left panels) of rectal carcinoid (**A, B**) and ileal carcinoid (**C, D**), respectively. Right panels show negative control of the same area in a consecutive section. Arrowheads and boxes indicate labeling. Boxed areas shown in higher magnification to illustrate cells displaying dense labeling. Scale bar = 50 μm .

Figure 1

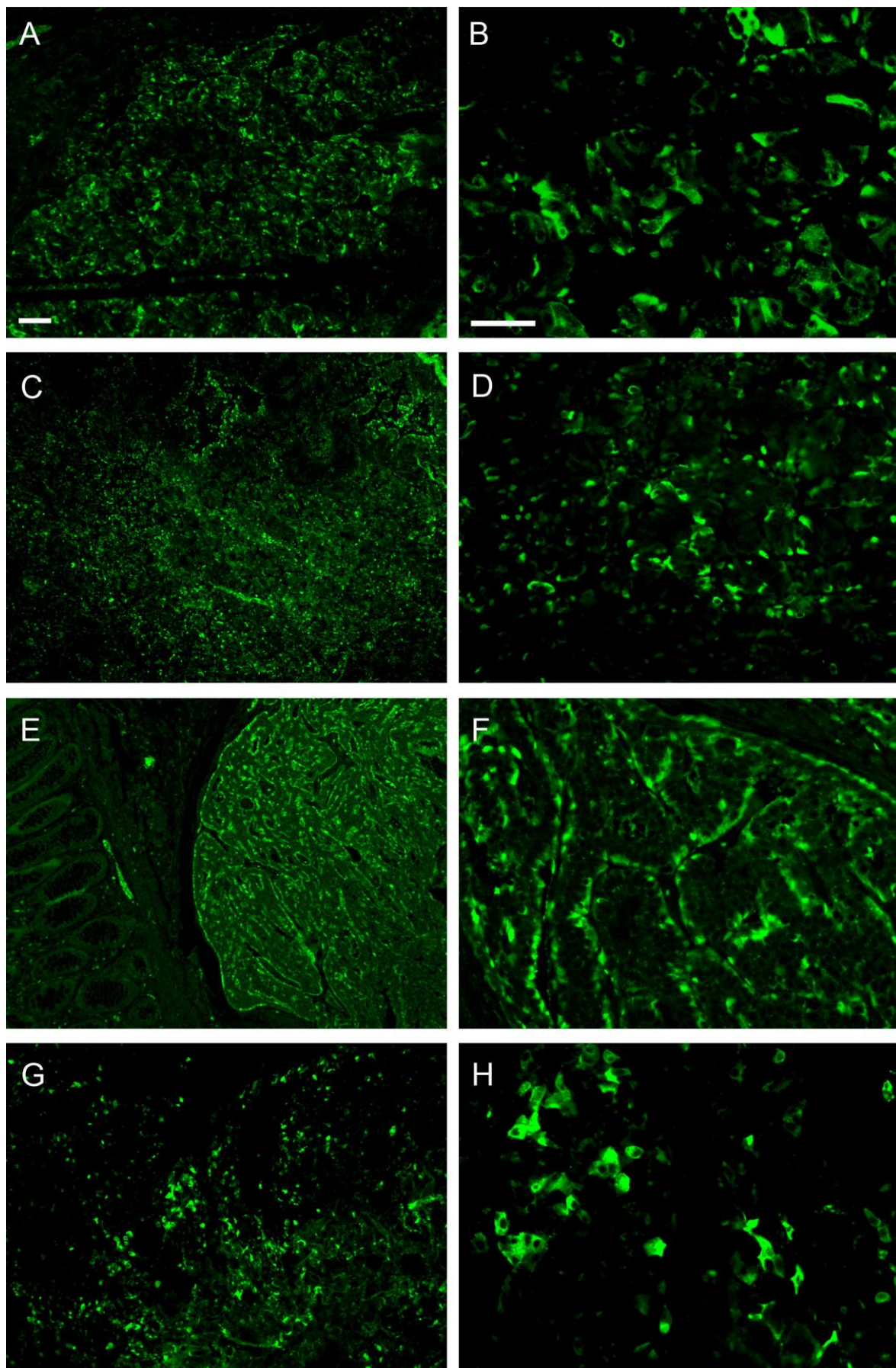


Figure 2

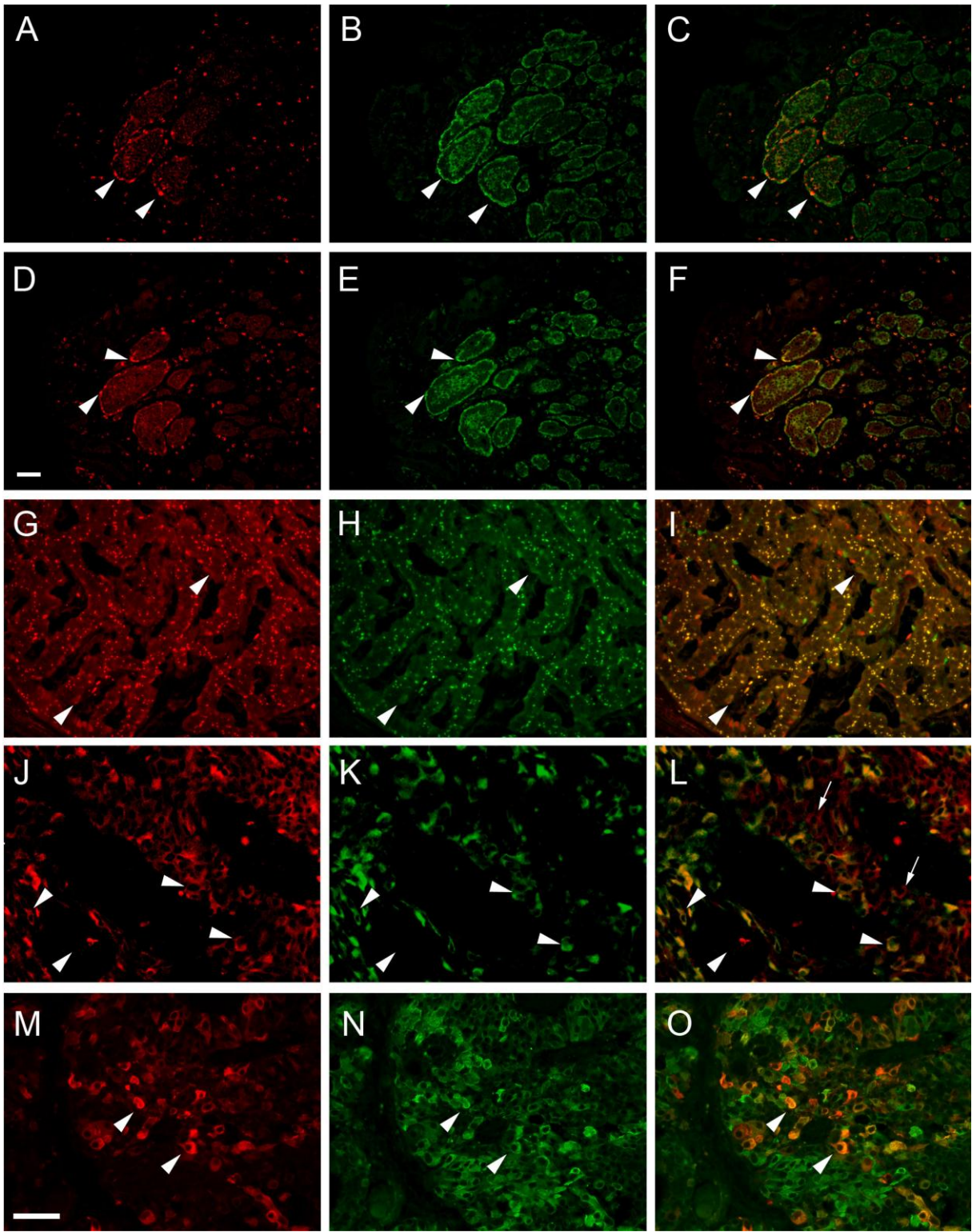


Figure 3

