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**Gaetano Magro · Martino Ruggieri**
**Filippo Fraggetta · Sebastiano Grasso**
**Giuseppe Viale**

# Cathepsin D is a marker of ganglion cell differentiation in the developing and neoplastic human peripheral sympathetic nervous tissues

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**Abstract** Cathepsin D (CD) is an aspartic proteinase which has been immunolocalised in intestinal ganglion cells of human neonates and adults. The aim of the present study was to define whether CD is a reliable ganglion cell differentiation marker in routinely fixed, paraffin-embedded tissues. For this purpose, we investigated immunohistochemically the expression and distribution of CD in the developing human peripheral sympathetic nervous system (PSNS) and gastroenteric nervous system (GENS), and in childhood neuroblastic tumours (NTs; neuroblastomas, ganglioneuroblastomas and ganglioneuromas), where ganglion cells differentiate from immature neuroblastic cells. During ontogenesis, CD expression is restricted to ganglion cell lineage with a progressively more intense cytoplasmic staining, mirroring the morphological differentiation of ganglion cells with increasing gestational ages. In neoplastic tissues, CD immunoreactivity was restricted to neuroblastic cells showing morphological features of gangliocytic differentiation (differentiating neuroblastomas, ganglioneuroblastomas) as well as to neoplastic ganglion cells (ganglioneuroblastomas, ganglioneuromas). We conclude that CD is a reliable ganglion cell differentiation marker, which can be used routinely to stain developing and mature ganglion cells in formalin-fixed, paraffin-embedded tissues. Furthermore, our results indicate that CD immunoreactivity in childhood NTs recapitulates the changes during normal PSNS development, as previously reported for Bcl-2 oncoprotein, c-ErbB2, insulin-like growth factor 2 and β2-microglobulin. This is consistent with the current view that childhood NTs exhibit gene expression profiles mirroring those occurring during PSNS ontogenesis.

**Keywords** Cathepsin D · Human fetus · Sympathetic nervous system · Neuroblastic tumours

G. Magro (✉) · S. Grasso
Istituto di Anatomia Patologica, Università di Catania,
Via Biblioteca 4, 95124, Catania, Italy
e-mail: magrog@dimtel.nti.it
Tel.: +39-95-310241, Fax: +39-95-316045

M. Ruggieri
Division of Pedriatic Neurology, Pediatric Clinic,
University of Catania, Italy
Department of Clinical Genetics,
Oxford Radcliffe Hospital NHS Trust, Oxford 0X3 7LJ, UK

G. Viale · F. Fraggetta
Department of Pathology and Laboratory Medicine,
European Institute of Oncology,
University of Milan School of Medicine, 20141 Milan,
Italy

## Introduction

Several antibodies to neurone specific enolase (NSE), protein gene product 9.5, microtubule-associated proteins, placental alkaline phosphatase, neuropeptide-Y, synapthophysin and peripherin are currently used to decorate ganglion cells immunocytochemically [9, 13, 35, 36, 38, 39], yet their specificity is often hampered by the concurrent staining of peripheral nerve fibres and other nervous cells.

Cathepsin D (CD) is an aspartic proteinase playing an important role in the intracellular protein catabolism [24], which has been localised to several normal and neoplastic human tissues. Apart from in macrophages and connective tissue cells, CD has also been found in epithelial cells of stomach [32, 34], colon [2, 32], breast [5, 32], endometrium [4], thyroid [22], bladder [10], bronchus [32], cornea [42], retina [42], choroid plexus [33] and in pneumocytes [20], hepatocytes [32] and neurons [3, 29, 33]. Among neoplastic tissues, CD is mainly expressed in gastric [34], colo-rectal [2], breast [5], endometrial [30], bladder [10], thyroid [22], skin [21], kidney [32], ovary [32], pancreas [32] cancer and in a wide variety of central nervous system neoplasms [33]. CD immunoreactivity has been recently detected in intestinal ganglion cells of submucosal and myoenteric plexuses of human neonatal and adult large intestine, but it is consistently absent from nerve fibres. Accordingly, this proteinase has been suggested as a possible ganglion cell marker, potentially useful in the diagnosis of Hirschsprung's disease [1].

The current study was aimed at ascertaining whether CD is a reliable ganglion cell differentiation marker in routinely

processed formalin-fixed, paraffin-embedded tissues. For this purpose, the distribution of CD immunoreactivity was analysed in the developing human peripheral sympathetic nervous system (PSNS) and gastro-enteric nervous system (GENS), whereby ganglion cells differentiate from a common neural crest-derived cell precursor [8, 11].

Several studies support the concept that childhood neuroblastic tumours (NTs; especially neuroblastomas) recapitulate morphologically and immunophenotypically the different developmental stages of the PSNS [16, 17, 18, 23, 25, 40, 41]. Thus, we have also investigated CD immunoreactivity in these tumours, comparing it with that in fetal tissues. We are not aware of any previous report on CD expression and distribution in the developing human PSNS and GENS, while only one immunohistochemical study has been reported on childhood NTs [31].

## Materials and methods

### Fetal and neonatal tissues

We have investigated immature neuroblastic cells, developing ganglion cells and developing chromaffin cells in fetal and neonatal paravertebral, pre- and peri-aortic ganglia and paraganglia, in the submucosal and myoenteric nervous plexuses and in the adrenal gland. Tissue samples were collected from 20 human fetuses ranging from the 8th to the 12th week of gestational age (wGA) obtained from legal interruptions [26]. Fetal developmental age was based on size, including crown–heel, crown–rump and heel–toe measurements [26, 37]. Twenty-two fetal and neonatal adrenal glands (aged from the 15th wGA to 2 years after birth), four stomachs and three large intestines (from the 15th wGA to 2 months after birth) were also investigated.

All tissue samples were fixed in 10% neutral buffered formalin for 12 h and embedded in paraffin. Sections stained with haematoxylin and eosin were checked histologically to exclude pathological changes.

### Adult tissues

Seven normal adult adrenal glands with periadrenal sympathetic ganglia were obtained from patients undergoing nephrectomy for renal cell carcinoma. Gastrointestinal submucosal and myoenteric nervous plexuses were investigated in ten total gastrectomies or colorectal resections for carcinoma and in five ileal resections for Crohn's disease.

### Neuroblastic tumours

Eight neuroblastomas (one undifferentiated and seven differentiating neuroblastomas) of adrenal glands in infants aged from 8 months to 2 years, five ganglioneuroblastomas (four retroperitoneal and one of the superior mediastinum) in infants aged from 1 year to 2 years, and three ganglioneuromas (one adrenal, one of the posterior mediastinum and one retroperitoneal) in infants 3 years to 7 years old were included in the study. The histological diagnosis of the different tumour types was based on established morphological criteria [18, 19, 23].

### Immunohistochemistry

Immunohistochemical studies were performed using the standard labelled streptavidin-biotin technique, using commercially available reagents (LSAB kit, Dako, Glostrup, Denmark). Briefly, sections were dewaxed in xylene for 15 min, rehydrated and treated with 3% $H_2O_2$ for 10 min to block endogenous peroxidase activity, followed by a rinse in distilled water and a 15-min wash in 0.01 M phosphate-buffered saline (PBS), pH 7.4. Proteolytic digestion was performed with 0.01 trypsin (Sigma, Chemical Co., St Louis, Mo.) in PBS, pH 7.4, for 10 min at 37°C to enhance staining intensity.

Incubation with primary antibodies (polyclonal anti-cathepsin D; dilution 1:500; monoclonal anti-chromogranin A, diluted 1:300; and monoclonal anti-NSE, pre-diluted, all from Dako) was performed overnight at 4°C followed by incubation with the linking antibody (biotinylated anti-mouse immunoglobulins, Dako) and with the peroxidase-conjugated streptavidin (Dako) for 20 min at room temperature. Peroxidase activity was developed in the 3,3'-diaminobenzidine (Sigma) substrate with 0.01% $H_2O$ for 5 min. Slides were counterstained with haematoxylin, dehydrated and mounted. Positive controls were performed on selected strongly positive cases of human normal liver and gastric mucosa [32]. Negative controls sections were incubated in PBS in place of the primary antibodies.

## Results

### Normal tissues

During the early phases of development (from the 8th to the 12th wGA), clusters of primitive sympathetic neuroblasts (round or oval cells with a tiny cytoplasmic rim and hyperchromatic nuclei with numerous nucleoli) interconnected by nerve fibres, were detected from the paravertebral regions to adrenals (Fig. 1A). These cell clusters colonised the adrenal glands and were found throughout the adrenal cortex to the central veins of the deep regions (Fig. 1B). From the 28th to the 38th wGA, these immature cell clusters progressively decreased in number until disappearance in neonatal adrenals. Throughout development, the neuroblasts were stained with NSE but did not show any CD immunoreactivity (Fig. 1A, B).

From the 8th wGA within the immature neuroblastic cell clusters, some larger cells – most likely developing (immature) ganglion cells – were immunostained for CD (Fig. 2A). In older fetuses (from the 12th to the 38th wGA), a steadily increasing granular to diffuse cytoplasmic staining for CD was detected in these developing ganglion cells in the preaortic, paravertebral and periadrenal ganglia, in the adrenal medulla, and in submucosal and myoenteric nervous plexuses of the gastrointestinal tract (Fig. 2B, C). The fully differentiating ganglion cells were recognisable for the progressive cell enlargement and the vesicular nucleus with one or more prominent nucleoli (Fig. 2B–D). CD immunoreactivity was maintained in ganglion cells of neonatal and adult sympathetic ganglia, adrenal glands, and gastrointestinal nervous plexuses (not shown). Schwann cells of nerve fibres associated with ganglion cells lacked any CD immunoreactivity (Fig. 2D). CD immunostaining was also detected in the cytoplasm of

**Fig. 1** Peri-preaortic (A) and intra-adrenal (B) clusters of undifferentiated neuroblasts (*N*), in human fetuses of 10 weeks and 12 weeks of gestational age (wGA), respectively, are not stained for cathepsin D (CD). Cytoplasmic immunoreactivity for CD is, however, shown by the adrenocortical cells (B) surrounding neuroblasts. A paravertebral paraganglion of a 15-wGA human fetus is stained with chromogranin A (C), but it is unreactive for CD (D) in consecutive sections. Original magnifications, **A** ×100; **B** ×250; **C, D** ×125 ▶

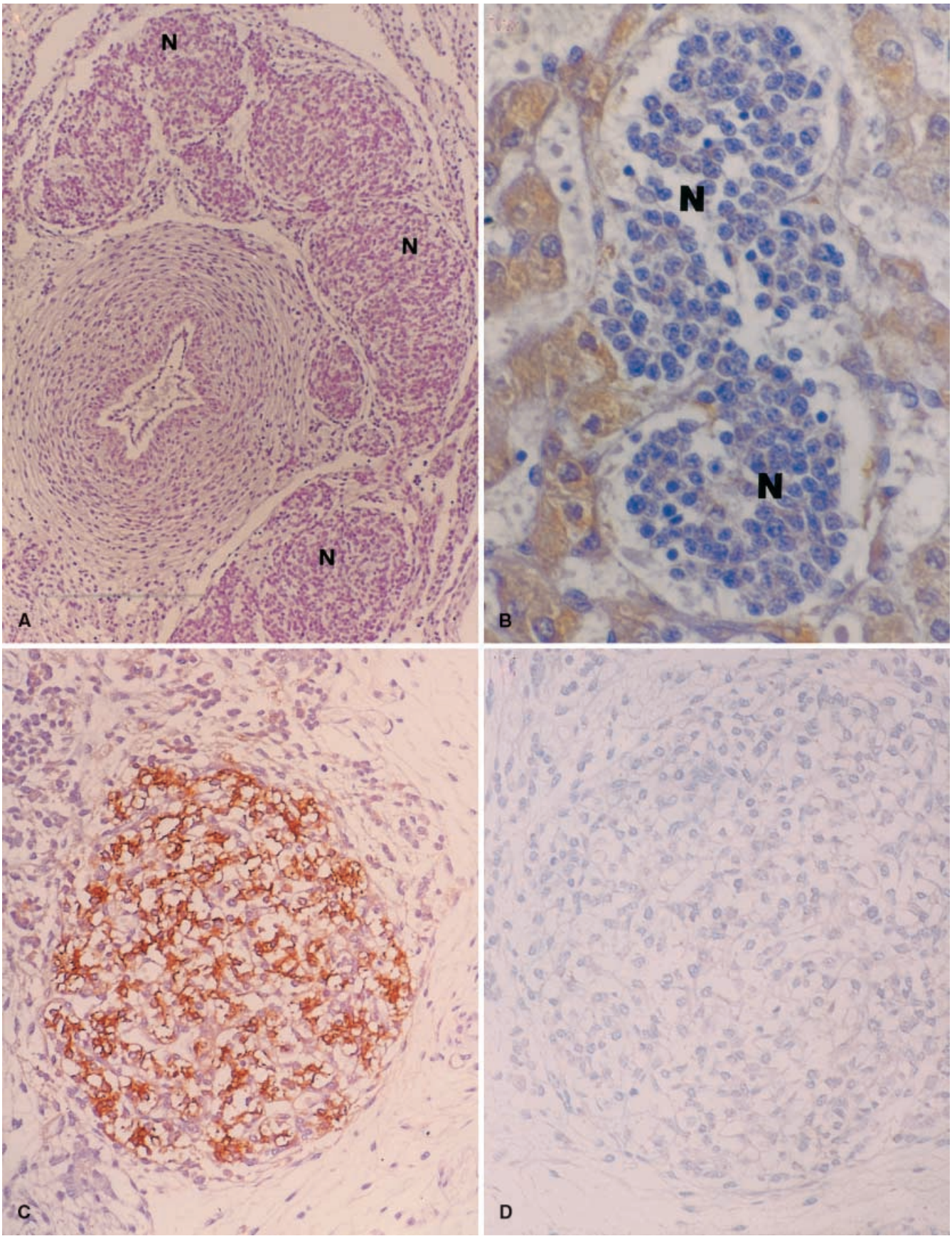



**Fig. 1A–D**

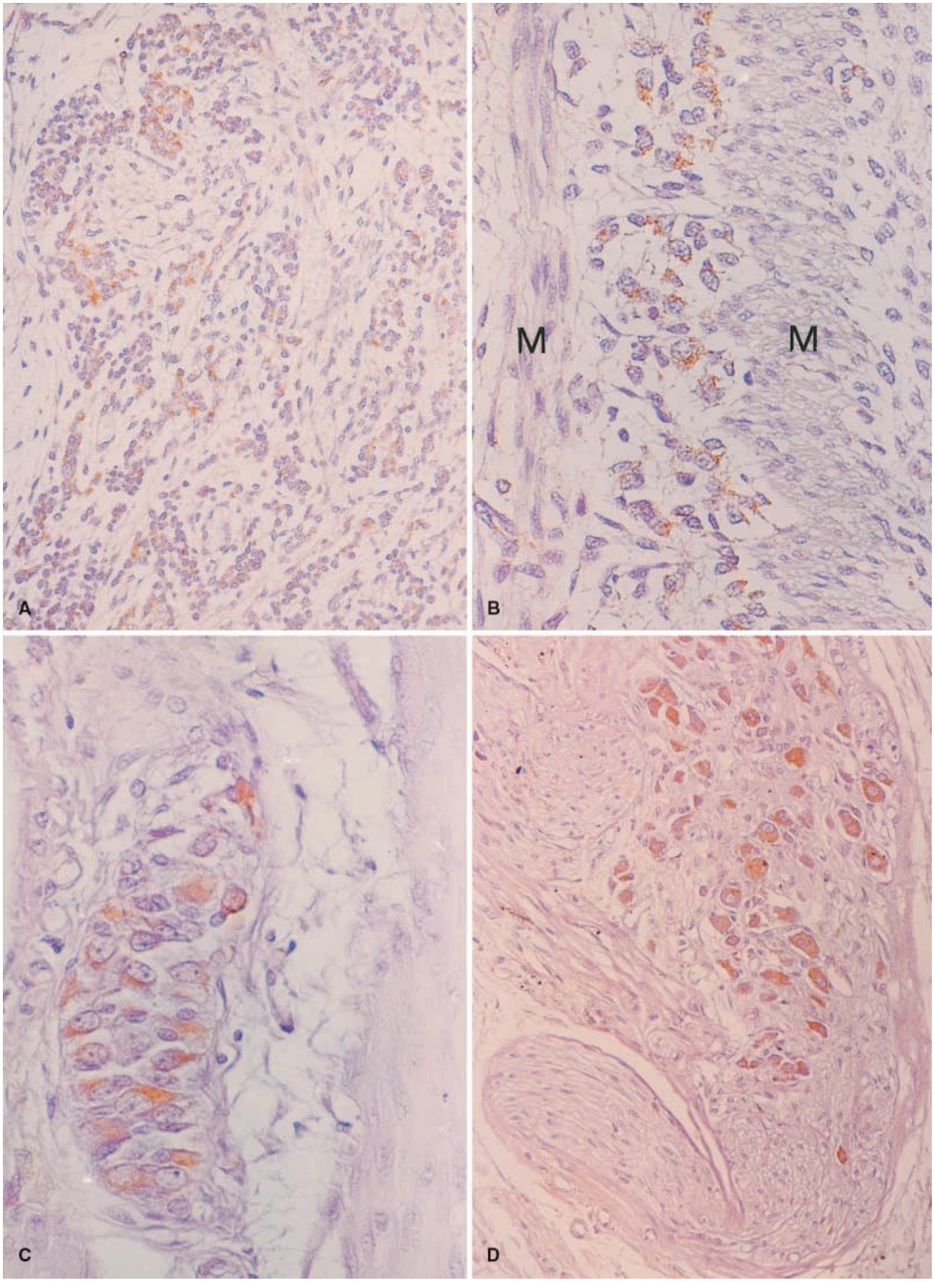



**Fig. 2A–D**

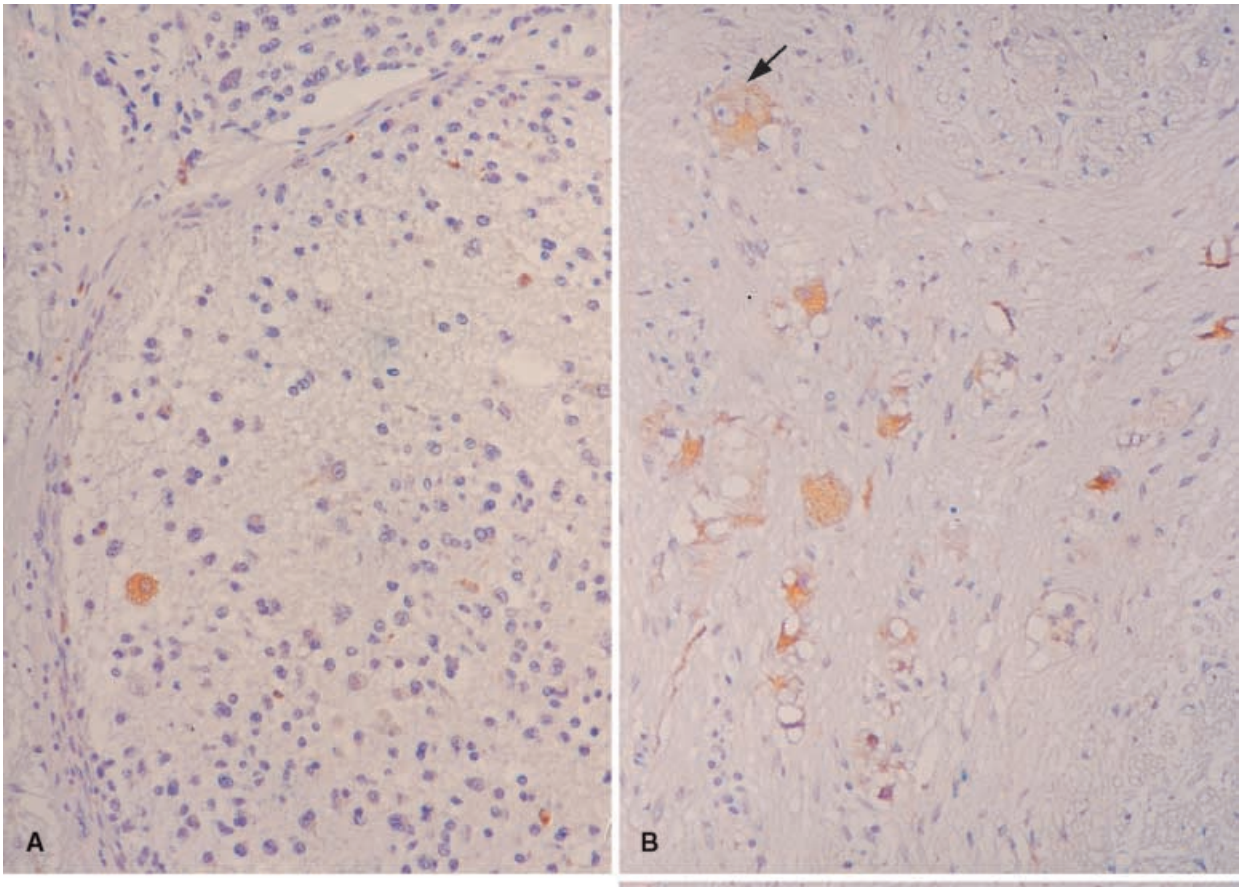
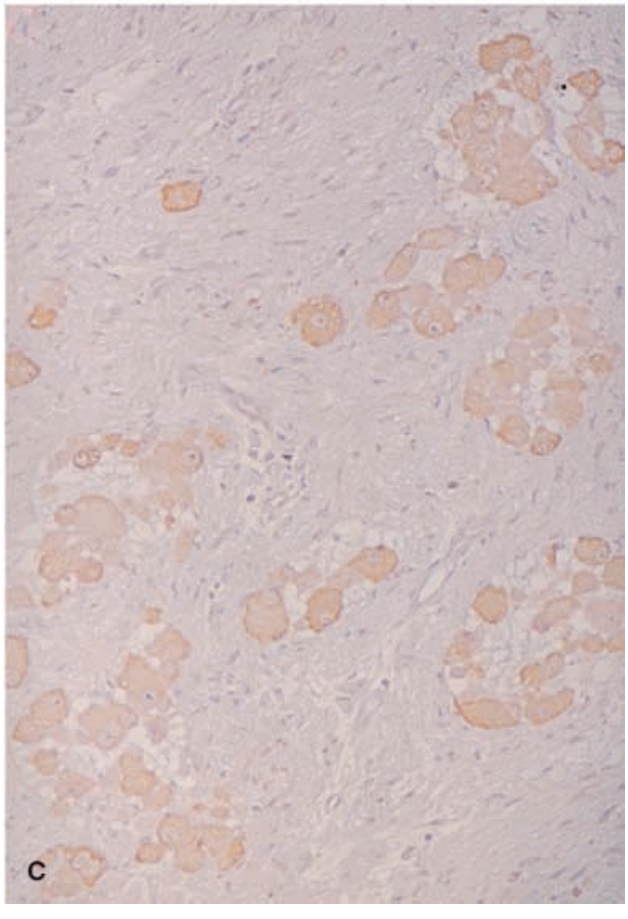



**Fig. 3 A** Differentiating neuroblastoma. Neuroblasts showing morphological features of gangliocytic differentiation are stained for cathepsin D (CD). In the undifferentiated neuroblasts, CD is absent or only focally expressed. **B** Ganglioneuroblastoma, borderline type. The *depicted area* is composed by rare mature ganglion cells (*arrow*) and neuroblastic cells showing a variable degree of ganglion cell differentiation (immature ganglion cells), interspersed within a Schwannian stroma. Both mature and differentiating ganglion cells show CD immunoreactivity, while Schwann cells do not. **C** Neoplastic ganglion cells of a ganglioneuroma are immunoreactive for CD. Original magnifications, **A–C** ×125




◀ **Fig. 2 A** Most cells of a paravertebral cluster of neuroblasts in a 12-week of gestational age (wGA) fetus exhibit cytoplasmic immunoreactivity for cathepsin D (CD). **B** Nervous cells with morphological features of immature ganglion cells and with a distribution within the smooth muscle layer (*M*) typical of the developing myoenteric nervous plexus are stained with CD in the rectum of a 15-wGA fetus. **C** Ganglion cells of the gastric myoenteric nervous plexus in a 34-wGA fetus show strong cytoplasmic CD immunoreactivity. **D** Ganglion cells of a peri-adrenal ganglion of a 36-wGA fetus are strongly positive for CD, whereas Schwann cells are unstained. Original magnifications, **A** ×125; **B** ×320; **C** ×400; **D** ×100

the developing adrenocortical cells (Fig. 1B) surrounding the clusters of undifferentiated neuroblasts, and it was maintained in neonatal and adult adrenal cortex.

From the 8th wGA, differentiating adrenal and extra-adrenal (sympathetic ganglia and paraganglia) chromaffin cells were identifiable for their chromogranin A immunoreactivity (Fig. 1C). In the adrenals, these cells were closely associated with the primitive neuroblastic cell clusters, as individual cells or small nests. They progressively increased in number and size from the 28th wGA to develop the adrenal medulla. Extra- and intra-adrenal immature chromaffin cells were not stained with CD (Fig. 1C, D), while adult adrenal medullary chromaffin cells showed focal and weak CD immunoreactivity.

### Peripheral neuroblastic tumours

The morphologically undifferentiated neuroblasts in neuroblastomas and ganglio-neuroblastomas did not exhibit any CD immunostaining (Fig. 3A). This was detected in the cytoplasm of neuroblasts showing morphological evidence of gangliocytic differentiation (cytoplasmic and nuclear enlargement, cytoplasmic eosinophilia, tumour giant cells with a single large or multiple nuclei; Fig. 3A, B), as well as in the ganglion cells of both ganglioneuroblastomas and ganglioneuromas (Fig. 3B, C). Supportive spindle cells surrounding tumour cell nests were also stained in some areas.

## Discussion

Several morphological, immunohistochemical and in vitro studies indicate that childhood NTs recapitulate the subsequent developmental stages of normal PSNS [6, 7, 16, 17, 19, 23, 25, 41]. This has prompted the search for specific cell differentiation markers [12, 14, 15, 16, 25, 26, 27, 28] suitable for diagnostic purposes [23] and for a better understanding of the biology of NTs [16, 17]. The focus on ganglion cell differentiation is of special interest because the extent of gangliocytic differentiation is one of the most reliable parameters for the classification and the prognostic evaluation of NTs [18, 19, 23].

Investigations on developing human PSNS and GENS, which arise from a common neural crest-derived precursor cell [8, 11], allow the pathway of ganglion cell differentiation to be followed, and have shown that it is characterised by the appearance of a distinct immunophenotype [16, 17]. However, markers of ganglion cells are usually not specific to this cell lineage because they are also shared by chromaffin cells (tyrosine hydroxylase, CD44, NSE) and neuroblasts (neuropeptide-Y, HNK-1/N-CAM, Bcl-2) [16, 17]. Recently, CD has been immunolocalised in the intestinal ganglion cells of human neonates and adults, and it has been considered as a specific cell marker, suitable for diagnostic purposes in routinely processed tissues [1].

We have investigated the developmentally regulated expression and distribution of CD in human PSNS and GENS, and compared the results with those obtained in childhood NTs. During PSNS and GENS development, CD immunoreactivity is restricted to ganglion cell lineage, whereas undifferentiated neuroblasts and developing chromaffin cells remain consistently unstained. CD immunoreactivity parallels the morphological differentiation of ganglion cells, as documented by a progressively more intense cytoplasmic staining of the developing ganglion cells with increasing gestational ages. CD immunoreactivity is also maintained in the ganglion cells of sympathetic ganglia and GENS in neonates and adults.

In infantile NTs, CD immunoreactivity is restricted to neuroblastic cells showing morphological evidence of ganglion cell differentiation (differentiating neuroblastomas, ganglioneuroblastomas) and to the mature ganglion cells of both ganglioneuroblastomas and ganglioneuromas. These findings confirm previous observations [31] of CD immunoreactivity in neuroblasts showing gangliocytic differentiation, and in neoplastic ganglion cells of neuroblastomas/ganglioneuroblastomas and ganglioneuromas, respectively.

The comparative evaluation of the immunohistochemical findings in fetal and neoplastic tissues indicates that CD expression in childhood NTs mirrors its normal developmental regulation in PSNS, as already reported for Bcl-2, c-ErbB2, insulin-like growth factor 2 and β2-microglobulin [7, 12, 14, 25]. This strongly supports the view that infantile NTs arise from a disturbed and/or blocked differentiation process at different stages of the PSNS ontogenesis [6, 16, 17, 18, 23, 40]. The role of CD expression in developing and mature ganglion cells and whether it is directly involved in ganglion cell differentiation remain to be elucidated.

In conclusion, although CD is widely expressed in a variety of normal and neoplastic human tissues, including the developing and mature adrenocortical cells as shown in the current study, this proteinase is a reliable ganglion cell differentiation marker in the human PSNS and GENS, as well as in childhood NTs. It may be particularly useful in the diagnosis of developmental abnormalities of the enteric nervous system (Hirschprung's disease and neuronal intestinal dysplasia), as previously suggested [1], and in the assessment of the extent of gangliocytic differentiation in NTs.

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