The Nerve-Cancer Connection in Ovarian Cancer

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Thesis submitted to the School of Biomedical Sciences and Pharmacy, University of Newcastle, as part of

Doctor of Philosophy in Human Physiology

April 18

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ABSTRACT

Background: Ovarian cancer is usually asymptomatic or the symptoms appear at advanced stages. Despite improvements in treatments, patient survival remains around 40% at 5 years. Ovarian tumours are histologically categorized into serous, mucinous, endometrioid, and clear cell carcinomas. Cervical cancer is the fourth most common female cancer, and affects the cells of the uterine cervix. Squamous cell carcinomas and adenocarcinomas constitute the main histopathological types of cervical cancer and have been strongly linked to specific HPV infections.

Progression of tumours is dependent on both the genetic makeup of cancer cells and the surrounding environment - the tumour microenvironment. The tumour microenvironment consists of cells providing structural support, vascular supply, immune cells and a range of growth factors. It has recently been shown that nerve fibres can infiltrate the tumour microenvironment in several solid tumours. Moreover, cholinergic and/or noradrenergic autonomic nerves have been shown to be associated with poor prognosis in prostate, breast and gastric tumours. The role of nerves in gynaecological cancers has not been determined and this encapsulates the main aim of this thesis.

Results: First, we investigated nerve infiltration and the expression of nerve growth factor (NGF) in 202 ovarian cancers *vs.* 18 normal ovarian tissues. Axons were detected by immunohistochemistry using the pan-neuronal marker PGP9.5. Axons could be observed in 72% of normal ovarian tissues but in only 9% of ovarian cancers. Tyrosine hydroxylase, a marker of peripheral sympathetic nerves, was expressed in

89% of normal ovary tissues but only 7% of ovarian tumours. NGF was expressed in 11% of normal ovarian sections and 28% of ovarian cancers. No statistical association was found between the presence of axons, or NGF expression, and age, tumour histological type, grade or lymph node involvement.

Next, I used markers for cholinergic (acetylcholine releasing) and a population of sensory nerve fibres (Substance P releasing) to further define the nature of ovarian tumour innervation. Cholinergic innervation was found to be prominent in the ovarian tumour microenvironment while substance P was a rare feature in both normal and ovarian cancer.

I also considered the presence of nitrigic axons in the ovarian tumour microenvironment, as detected by the presence of nNOS. Although our nNOS antibody was specific for neurons in the gut many non-neuronal cells were labelled in ovarian cancer making analysis of the nNOS expression difficult.

I also investigated the presence of the high affinity NGF receptor, TrkA. While many tumours were TrkA-IR, there was no statistical correlation to clinicopathological parameters or NGF-IR. Instead, other neurotrophins receptors TrkB (BDNF preferred receptor) and $p75^{NTR}$ showed more common expression. Sortilin, receptor for pro-neurotrophins, on the other hand was not detected in the normal ovary and seldom expressed in ovarian tumours.

The receptors for the GDNF family – GFRs - were also studied. GFRα1, 2 & 3 were observed and described for the first time in normal ovary and ovarian tumours. All GFRs were detected in both normal and ovarian cancer. GFRα3 showed the most intense IR which may be linked to the antibody characteristics itself. However, all GFRs were detected in blood vessels layers, in *varicose* across the ovarian stroma and in the GC cells of ovarian follicles. Some ovarian cancer cells were also GFR-IR.

In order to further investigate the role of nerves and neurotrophic factor NGF in ovarian cancer *in vitro* migration and co-culture assays were conducted. My results showed that OVCAR-3 ovarian cancer cells cause 50B11 cells to grown neurites but this is not due to the action of proNGF or NGF. Thus, other neurotrophins as the GDNF family of trophic factors may be major component in the ovarian microenvironment.

In vitro Transwell assays with ovarian cancer cells OVCAR-3 showed that acetylcholine has the potential to affect ovarian cancer cells migration, but not NA or SP. Acetylcholine also had a larger effect in ovarian cancer morphology (OVCAR-3 cells numbers, size and shape).

Finally, I compared innervation of ovarian cancers to cancers of the cervix. Similarly, to ovarian tumours, I used TMAs of cervical tissue biopsies and screened them with the pan neuronal marker PGP9.5 and neutrophins proNGF and NGF. 93% (272 out of 294 samples) of cervical tumours showed PGP9.5-IR and 87% (257 out of 272) PGP9.5-IR tumours were innervated by axons. No correlation was observed between innervation and clinopathological parameters except for higher PGP9.5-IR in patients under 50 years old. All cervical cancer types were highly innervated (>90% of respective samples). Neuroendocrine cells PGP9.5-immunopositive were also detected in tumours of the cervix. NGF and pro-NGF expressions were increased in cervix tumours. Moreover, we found significant more NGF and proNGF-immunoreactivities in cervix tumours compared with normal cervix. Higher grade tumours also showed more NGF and proNGF expression. Thus, while there was no clear association between axons and neurotrophin expression, the later was significant and may not be required for cervix innervation maintenance or neo-development during cancer progression.

Conclusion: Together, these results indicate that nerve infiltration is occasionally found in the tumour microenvironment of ovarian cancer. Cholinergic innervation is predominant over adrenergic nerves. Sensory Substance P axons are a rare feature but ovarian cancer cells express its main receptor, NK1. Moreover, ovarian cancer cells stimulate neurite outgrowth of nociceptive sensory neurons, and cancer cell migration and morphology can be affected by neurotransmitters. Nerve infiltration is a prominent feature of the cervical tumour microenvironment. This is perhaps not surprising given the generally dense innervation of the normal cervix. Moreover, cervix tumours highly expressed neurotrophins NGF and proNGF, particularly in higher grade cancers. Thus, anti-neurotrophin treatments might still be a therapeutic option for cervical cancer patients.

Overall the data suggest that the impact of nerves on gynaecological cancers may be different to other solid tumours.

Keywords: ovarian cancer, tumour microenvironment, axons, TH, cholinergic, peptidergic, NGF, Substance P, vAChT, NK1, Trk receptors, OVCAR-3, 50B11, cervical cancer

DECLARATION

I hereby declare that this submission is my own work and to the best of my knowledge, it does not contain materials previously published or written by another person, nor has material in this thesis been accepted for the award of any other degree at the University of Newcastle or another educational institute. Any contribution made to the research by others, with whom I have worked at the University of Newcastle is explicitly acknowledged in this thesis.

Sonia M. Rodrigues Oliveira

ACKNOWLEDGEMENTS

Most of all I would like to thank my principal advisor, Dr Phillip Jobling for all the support, mentorship and his persistent guidance in what has been a challenging and fulfilling journey. For his invaluable knowledge in neurophysiology, electrophysiology, statistics and professional and personal assistance. Thank you for leading me in this adventure.

To Professor Hubert Hondermarck, for his creative perspectives, his in-depth reviews, for his eye to detail, thorough knowledge of laboratory protocols and broad knowledge in cancer research, and for allowing me the opportunity to challenge my scientific and professional views.

I would like to thank Dr. Rick Thorne, Sam Faulkner and Dr. Jay Pundavela for their assistance. Dr. Pradeep Tanwar, Annie Ko and Preety Bajwa for providing the OVCAR-3 cells. Professor Rainer Haberberger for supplying the 50B11 immortalised sensory neurons.

To all the valuable advice from students, laboratory assistants, staff and academics from the School of Biomedical Sciences and Pharmacy, in particular to Dr Severine Roselli for her input in histology protocols and to Professor Phil Bolton for the valuable and comprehensible knowledge in biostatistics.

To my friend, Marina Mišikić for her friendship, support, care and for always been there for me through the ups and downs of this long and rewarding road.

I am nothing.
I'll never be anything.
I couldn't want to be something.
Apart from that, I have in me all the dreams in the world.
— Fernando Pessoa

Finally, thank you, once again to all the people that I have believed in me, in particular my Mother Lurdes Oliveira who has enabled my dreams and knowledge to entwine and has supported me and, the dreams I now walk into my future with.

This thesis is dedicated to the memory of my father, António Marques de Oliveira. He is missed every day, and without his support and fatherly encouragement I probably wouldn’t be the person and the scientist I am today.

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LIST OF ABBREVIATIONS AND ACRONYMS

-IR, immunoreactive / immunoreactivity

ACh, acetylcholine

AIHW, Australian Institute of Health and Welfare

AOI, area of interest

ARG, aorticorenal ganglia

ARTN, artemin

BDNF, brain derived neurotrophic factor

BMP, bone morphogenic proteins

BV, blood vessel

CCC, clear-cell carcinoma

CG, celiac ganglion

CGRP, calcitonin gene-related peptide

CHAT, choline acetyltransferase

CNS, central nervous system

CO_2, carbon dioxide

DA, dopamine

DAB, 3’3’diaminobenzidine

DNA, deoxyribonucleic acid

DOPA, dopamine or 3,4-dihydroxyphenethylamine

DRG, dorsal root ganglia

EC, endometrioid carcinoma

ECM, extracellular matrix

ENK, enkephalin

Eph, ephrin(s)

ERα, estrogen receptor-α

FCS, foetal calf serum

FIGO, the International Federation of Gynaecology and Obstetrics

FSH, Follicle-Stimulating Hormone

FSHR, FSH receptors

G-CSF, granulocyte colony-stimulating factor

GABA, γ-Aminobutyric acid

GAL, galanin

GC, granular cells

GDNF, glial cell line-derived neurotrophic factor

GFL, GDNF family ligand

GFR, GFL receptor

GI, gastrointestinal tract

GnRH, gonadotropin-releasing hormone

Gt, goat

HER2+, human epidermal growth factor receptor 2-positive

HG, high-grade

HGEC, high-grade endometrioid carcinoma

HGSC, high-grade serous carcinoma

HN, hypogastric nerves

HPV, human papilloma virus

HRP, horseradish peroxidase

IB4, isolectin B4 from *Griffonia simplicifolia*

IGFs, insulin-like growth factors

IHC, immunohistochemistry

IL-1β, interleukin-1 beta

IMG, inferior mesenteric ganglion

LG, low-grade

LGEC, low-grade endometrioid carcinoma

LGSC, low-grade serous carcinoma

LH, luteinizing hormone

m, mouse

MC, mucinous carcinoma

mRNA, messenger RNA

NA, noradrenaline

NANC, non-adrenergic/non-cholinergic neurotransmitters

NE, norepinephrine

NGF, nerve growth factor

NHS, normal horse serum

NK1, Neurokinin 1

NKA, Neurokinin A

NKB, Neurokinin B

nNOS, neuronal NOS

NO, nitric oxide

NOS, nitric oxide synthase

NPY, neuropeptide Y

NRTN, neurturin

NT-, neurotrophin receptor-

NTRK, Neurotrophic receptor tyrosine kinases

OD, optical density

OPN, ovarian plexus nerve

OvCa, ovarian cancer

p53, tumour protein p53

$p75^{NTR}$, p75 neurotrophin receptor

PACAP, pituitary adenylate cyclase activating polypeptide

PBS, phosphate buffered saline

PC-3, human prostate cancer cell line 3,

PG, pelvic ganglion / ganglia

PGE2, prostaglandin E2

PGP9.5, protein gene 9.5

PP, pelvic plexus

proNGF, nerve growth factor precursor

PSPN, persephin

PVA, prechiasmatic area

Rb, rabbit

RET, tyrosine kinase membrane receptor for rearranged during transfection

Robo, roundabout

RNA, ribonucleic acid

SC, serous carcinoma

SC, serous carcinoma

SCJ, squamocolumnar junction

SD, standard deviation

SEM, standard error of the mean

SF, serum free

Sh, sheep

SN, splanchnic nerves

SOM, somatostatin

SON, superior ovarian nerve

SP, Substance P

SRG, suprarenal ganglion / ganglia

SRY, sex-determining region of the Y chromosome

TH, tyrosine hydroxylase

TMA, tissue microarray

TNM, staging system of malignant tumours (T, primary tumour size; N, nearby lymph nodes involvement; M, distant metastasis)

Trk, tyrosine kinase receptor

TrkA, tyrosine receptor kinase A or tropomyosin receptor kinase A

TrkB, tyrosine receptor kinase B or tropomyosin receptor kinase B

TrKC, tyrosine receptor kinase C or tropomyosin receptor kinase C

vAChT, vesicular acetylcholine transporter

VEGF, vascular endothelial growth factor

VIP, vasoactive intestinal polypeptide

WT, wild-type

CHAPTER 1: GENERAL INTRODUCTION

1.1. The ovary

Introduction

The ovaries are primary sexual organs that undertake their position in the lateral wall of females' pelvis after complete embryonic differentiation. Each of the two ovaries is hanging from the uterine horn by the ovarian ligaments. The ovaries are lined by a cuboidal epithelia layer named germinative superficial epithelia. This layer is separated from the adjacent stroma by a basal membrane that merges with the tunica albuginea, a white sheet of dense and irregular conjunctive tissue. The tunica albuginea encases the ovary and has a structure that allows its easy rupture during ovulation (Yoshimura and Wallach 1987, LeMaire 1989, Morioka, Zhu et al. 1989). The germinative epithelia, the basal membrane and the albuginea form the ovarian serosa, which resembles the serosa of other organs. Two zones are identified in the ovarian stroma: the cortex – the functional part of the ovary, under the albuginea and includes the ovarian follicles; and the medulla – majorly lax connective tissue with blood vessels, lymph vessels and axons or nerve fibres that arise from the ovarian hilum. The gross anatomy of the female reproductive tract and position of the ovary is represented in figure 1.1.

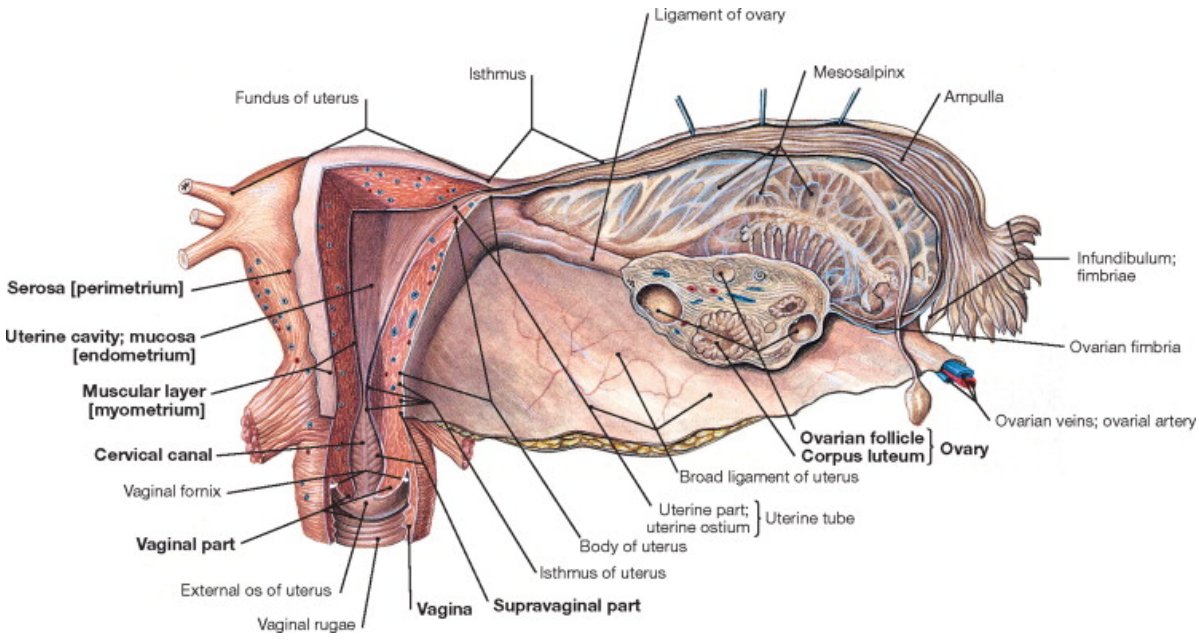


Figure 1.1- Gross anatomy of the female reproductive tract showing relative positions of ovary, fallopian tubes uterus cervix and vagina. From (Sobotta, Putz et al. 2006).

Ovarian innervation has been reported for more than a century. Sympathetic, parasympathetic and sensory nerves are reported to innervate the mammalian ovary and their neurotransmitters to influence the ovarian function (Kulkarni, Wakade et al. 1976, Malishevskaia and Brindak 1980, Mayerhofer, Dissen et al. 1997, Jana, Dzienis et al. 2007, Uchida and Kagitani 2014). Nonetheless, the complete anatomy and physiology of the autonomic nervous system in the female gonad remains incomplete and controversial. Experimental evidence seems to rest on species, developmental stage of the ovary and phase of the ovarian cycle. These make it difficult to fully assess and treat ovarian disorders.

1.2. Early development and differentiation of the ovary

The ovary is a multi-compartmentalized structure of unique and variable biological properties. It produces and releases its own hormones (progesterone, estrogens (mainly oestradiol), inhibin, and androstenedione) and cyclically produces an oocyte that upon sexual maturation determines the continuation of the species.

In mammals, gonads start to form during the first undifferentiated stage of embryogenesis, between the 3rd and 6th week of gestation (Larsen 2009). At this stage, there is no morphological difference between the genders. That happens during the 6th to 8th week of embryonic development, depending on the chromosomal pool of the embryo and, with the internal genitals differentiating first. Between the 10th and the 12th weeks of the embryonic development, androgens and sexual chromosomes transduction lead to the development of the sexual organs. In the absence of the Y chromosome, the female development occurs. This is because the somatic sex cord cells do not contain a Y chromosome or SRY region (sex-determining region of the Y chromosome), do not produce SRY protein and therefore do not differentiate into Sertoli cells. Consequently, Anti-Müllerian hormone, Leydig cells and testosterone are not produced and the male development of the genital ducts and accessory sexual organs is not stimulated thus ensuing the female development (Rey, Josso et al. 2000, Larsen 2009, Warr and Greenfield 2012). The mesonephric ducts degenerate and the paramesonephric ducts give rise to the fallopian tube, uterus and superior vagina. The close physical contact between the genital ridge and the mesonephros in females seems to play a role in inducing the initial stages of gamete maturation (Rey, Josso et al. 2000, Warr and Greenfield 2012, Makiyan 2016).

In the presumptive ovary, the primitive sex cords degenerate and follicle cells stem from the secondary cortical sex cords. These secondary sex cords then invest the primordial germ cells to form the follicle cells of the ovary. Thus, unlike that of the male development, the surface epithelium of the female gonad continues to proliferate giving rise to a second generation of cords (cortical cords) in the 7th week. The cortical cords then penetrate the underlying mesenchyme but remain close to the surface. In the fourth month, the cortical cords split into isolated irregular cell clusters with each surrounding one or more primitive germ cells in the medullary part of the ovary. These germ cells finally develop into oogonia and descendants of the surface epithelium form follicular cells. Germ cells differentiated into oogonia enter the first meiotic division as primary oocytes before they develop close interactions with the investing follicle cells. Later germ cells disappear from the medullary portion and are replaced by a vascular stroma that forms the ovarian medulla. The female germ cells enter meiosis but further nuclear development is inhibited by the follicle cells, which arrest germ cell development until puberty. Then, individual oocytes resume oogenesis in response to monthly surge of gonadotropins (LeMaire 1989, Larsen 2009, Makiyan 2016).

In summary, the intermediate mesoderm forms the longitudinal elevation along the dorsal body wall forms the urogenital ridge. The coelomic epithelia and mesoderm of urogenital ridge proliferate and form the gonadal ridge. The primary sex cords arise from the gonadal ridge and absorb in primordial germ cells from the yolk sac. Finally, the primary sex cords develop into *rete ovarrii* (that will absent in adulthood). And secondary sex cords develop and absorb in the primordial germ cells from the yolk sac. These break apart into irregular clusters that will form the primordial follicle cells and undergo folliculogenesis. Thus, the primary oocytes, composed of simple squamous

lining and ovarian stroma have mesodermic origin. Finally, the ovaries descend from the abdominal cavity into the pelvic cavity. This is potentiated by the gubernaculum, a fibrous tissue that runs from the abdominal wall to the end of the ovary forming the ovarian ligament, and to the labia majora forming the round ligament of the uterus (LeMaire 1989, Morioka, Zhu et al. 1989, Rey, Josso et al. 2000, Larsen 2009, Warr and Greenfield 2012, Makiyan 2016).

It is postulated that ovarian innervation develops before initiation of folliculogenesis and follow the formation of other vessels, as the blood vessels that enter the ovary through the ovarian plexus and the suspensory ligament (Aguado and Ojeda 1984, Malamed, Gibney et al. 1992). The sympathetic and afferent nerve fibres run with the ovarian ascending and vaginal branches of uterine vessels and connect with the pelvic plexus, forming the ovarian nerve plexus, and the peripheral nerve fibres arise from the pelvic splanchnic nerves following the same route as the ovarian blood and lymph vessels (Ojeda, White et al. 1983, Aguado and Ojeda 1984, Schultea, Dees et al. 1992, Ricu, Paredes et al. 2008).

1.3. Neural guidance molecules importance and peripheral nervous system development

Formation of peripheral ganglia and subsequent axonal outgrowth towards target tissues is under the control of a number of trophic factors, cell surface receptors and extracellular matrix molecules (Table 1.1) (Lowery and Van Vactor 2009). The earliest guidance molecules to be discovered are the “classical” neurotrophins as exemplified by the nerve growth factor (NGF) discovered by Levi-Montalcini and colleagues (Cohen, Levi-Montalcini et al. 1954). The neurotrophins consist of NGF, BDNF (brain derived neurotrophic factor), NT3 (neurotrophin 3), NT4/5 (neurotrophin 4 / 5) and the GDNF (glial-cell derived neurotrophic factor) family and their receptors (Table 1.1; Figure 1.2). NGF is synthesized by proteolytic cleavage of its precursor proNGF (Lessmann, Gottmann et al. 2003). proNGF has been reported abundant in central nervous system (CNS) where mature NGF is undetectable (Fahnestock, Yu et al. 2004). Plus, proNGF rather than NGF has also been detected in different cell types, including mast cells, sciatic nerve cells, thyroid gland, skeletal muscle, prostate gland, hippocampus and hair follicle (Hasan, Pedchenko et al. 2003). Originally, proNGF was assumed to be inactive however, now it is recognised that proNGF is biologically active, and it may have a different role to NGF in some circumstances (Fahnestock, Yu et al. 2004). For instance, it is generally held that proNGF can be neurotoxic by inducing a cascade of apoptotic responses when it binds to its preferred receptor $p75^{NTR}$ (p75 neurotrophin receptor) and, later this entity binds to a third receptor named sortilin. In contrast, NGF and its main receptor TrkA (tyrosine receptor kinase A) are known to induce neuronal survival/differentiation (Bradshaw, Pundavela et al. 2014) .

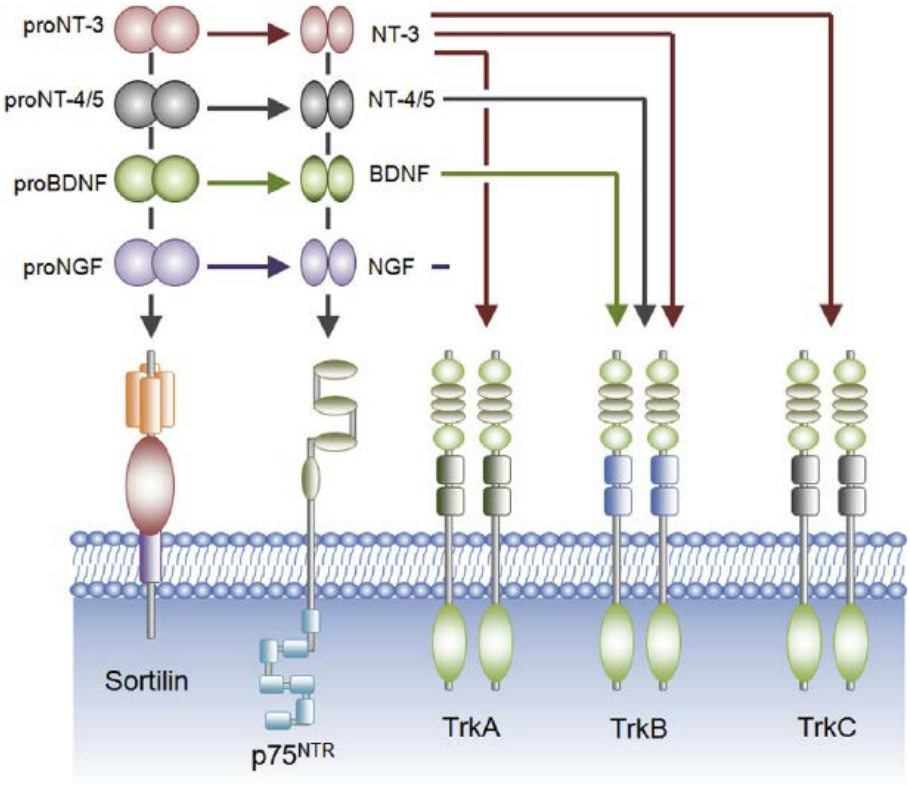


Figure 1.2 - Binding of neurotrophins and pro-neurotrophins to Trk receptors and p75^{NTR}. NGF, BDNF, NT-3, NT-4/5 as well as their respective precursors (proNGF, proBDNF, proNT, proNT-4/5) all bind to the pan-neurotrophin receptor p75 NTR (p75 neurotrophin receptor) while Trk (tirosine kinase) receptors bind neurotrophins with different specificities. Sortilin binds only the precursor forms. From (Bradshaw, Pundavela et al. 2015)

1.3.1. Formation of peripheral ganglia

It is postulated that nerve sprouting into tumours occurs in a similar manner to the normal axonal development during embryogenesis (Batista, Mariano et al. 2014, Brunet, Gordon et al. 2014, Dobrenis, Gauthier et al. 2015).

During neurulation, the neural crest cells originating in the neural folds migrate to specific locations in the body. Spinal neural crest cells migrate and give rise to autonomic preaortic ganglia, adrenal medulla and, dorsal root ganglia (DRG) and sympathetic paravertebral chain ganglia. The dorsal root ganglia house sensory neurons that are responsible for conducting the impulses to the spinal cord from end organs in the viscera, body wall and extremities. Neural crest cells migrate preferably through the rostral half of the sclerotomes. This is mediated by repulsive effects of epherins (Eph) that are expressed by cells in the caudal portion of the sclerotome and recognised by receptors expressed by these neural crest cells designated Eph tyrosine kinases. Survival and differentiation of the dorsal root ganglion are assumed to depend on brain–derived neurotrophic factor (BDNF) secreted by adjacent neural tube cells. These cells also secrete other growth factors, including bone morphogenic proteins (BMP). Other neurotrophic cues are also responsible for determining the anatomical location of the sympathetic and parasympathetic ganglia. Although the location of parasympathetic ganglia close to, or even within, visceral organs suggests that there are many differences between the two. In the thoracic, lumbar and sacral regions one pair of paravertebral chain ganglia forms in register with each pair of somites. Unlike DRGs, the chain ganglia are not dependent on BDNF for survival, but may depend on other growth factors such as insulin-like growth factors (IGFs) and NGF. Notably, not all peripheral sympathetic neurons are in the chain ganglia – some develop from neural crest cells that congregate next to major branches of the dorsal aorta.

1.3.2. Axon Guidance

Much of what we understand about axon guidance and growth cone function is derived from the CNS. Apical growth cones are the primary sites where new axons interact with their surroundings. The growth cones are thought to move by filopodia, guided to its destination by detecting molecular markers that delineate the correct route (pathfinding). Once the growth cones reach their target they halt and a synapse is formed. Somatic motor and sensory axons synapse directly with their target tissues.

Axons of the central autonomic neurons terminate in the peripheral autonomic ganglia where they synapse with the peripheral neuron of the “two-neuron” autonomic pathway. It is postulated that at a specific time during development the target tissue(s) release tropic substances that attract the correct growth cones or secrete a trophic substance that supports the viability of the growth cones that happen to take the “right” path. Trophic substances include netrin-1 and netrin-2 that were first implicated in the guidance of spinal cord commissural axons along a pathway from the dorsal cord to the ventral floor plate cells and in the guidance of retinal axons (Serafini, Colamarino et al. 1996, Brunet, Gordon et al. 2014, Xu, Wu et al. 2014). Interestingly netrins have also been implicated in reproductive development and fertility (Newquist, Hogan et al. 2013) and in malignancies including breast and ovarian cancers (Fitamant, Guenebeaud et al. 2008, Mehlen, Delloye-Bourgeois et al. 2011, Papanastasiou, Pampalakis et al. 2011).

The mechanisms underlying axonal growth and development have been well studied and a number of different guidance molecules have been identified (Bixby and Harris 1991, Chilton 2006, Canty and Murphy 2008) – Table 1.1.

Table 1.1 - Major families of molecules implicated in axon guidance. UNC: receptor for netrins; DCC: "Deleted in colorectal cancer", axon guidance receptor that responds to netrin-1; Eph: ephrin(s); NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor; NT, neurotrophin; GDNF: gial cell-derived neurotrophic factor; GFR: GDNF family receptors.

FAMILY	**RECEPTOR(S)**	**ACTION**
Netrins	UNC5 DCC	Repulsive via UNC Attractive via DCC
Semaphorins	Plexins Neuropilins	Neural repulsion
Slits	Robo (roundabout)	Neural repulsion
Ephrins	Ephs (14 members)	Neural repulsion / attraction
Neurotrophic factors		
- *NGF* - *BDNF* - *NT3, NT4/5*	Trk receptors $P75^{NTR}$	Neural attraction and survival
- *GDNF,* - *Artemin,* - *Neurturin*	GFR (GDNF receptors)	
Extracellular matrix molecules	Not one specific family.	Includes laminin, collagen, fibronectin (growth permissive), and chondroitin sulphate proteoglycan tenascin (growth inhibitory)

Two mechanisms have been suggested to drive axon growth towards a target: 1) contact guidance theory where the growing axonal tip is guided by a physical orientation of molecules or structures in the extracellular matrix and; 2) chemo-affinity

hypothesis where the growth cone shows different adherence to molecules that are distributed in the extracellular matrix such as fibronectin, laminin and, neural cell adhesion molecules. Likely it can be postulated that both these mechanisms may be occurring under the influence of tropic and trophic factors. The first or “pioneer” growth cone to traverse a route establishes a pathway later used by the growing axons

Figure 1.3 shows a representation of a growth cone and chemical cues present to attract and/or repulse its outgrowth. Finally, it should be remembered that despite different origins and locations of their somata, somatic motor, autonomic motor and sensory axons usually end up in mixed spinal or cranial nerves. The pattern of somatic motor and sensory innervation is segmental, i.e., based on the segmental organization established by the somites. In contrast, the pattern of sympathetic innervation is not entirely segmental. For example, the head receives sympathetic innervation via the cervical chain ganglia whereas the heart, trachea and lungs receive sympathetic innervation from cervical and thoracic chain ganglia.

A. **B.**

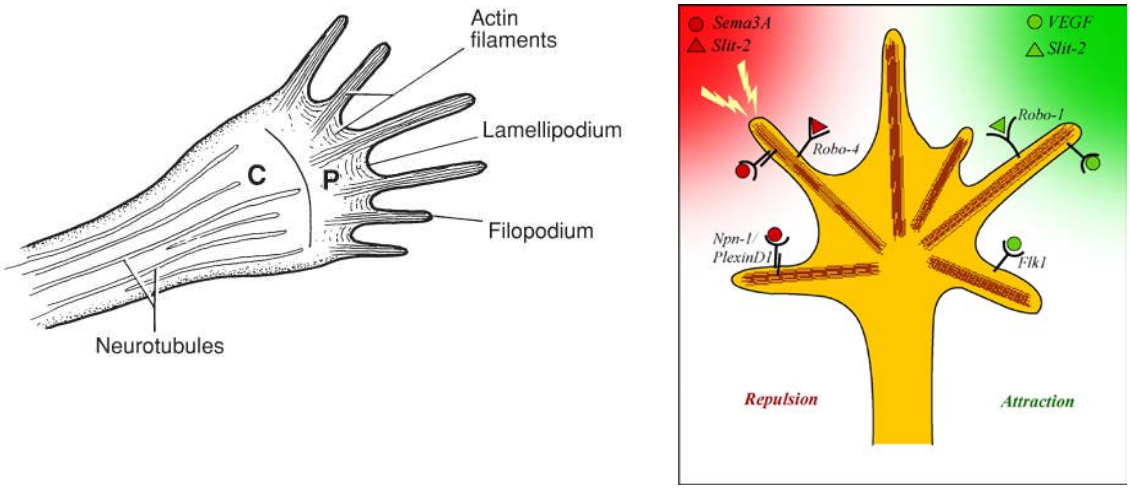


Figure 1.3 – (A) Growth cone. The growth cone is a specialized sensory motor structure at the growing terminal of an axon (neurite). Each growth cone comprises a central core domain (C) and a peripheral domain (P) from which extends the leading edge of finger-like protuberances called filopodia at the base of which are web-like veils called lamellipodia; these are shaped by actin filaments (one of the protein filaments of the cytoskeleton). Neurotubules are abundant in the organelle-rich central domain and the actin filaments predominate as tight bundles in the filapodia and as dense interwoven networks in the lamellipodia (Sanes and Jessell 2000); (B) Attractive and repulsive cues mediate the guidance of the endothelial tip cell growth cone. VEGF and Slit-2 act as attractant cues, via Flk1 and Robo-1, respectively, while Sema3A and Slit-2 act as repulsive cues, via Npn1/PlexinD1 and Robo-4, respectively. The involvement of Slit-2/Robo-4 axis in repulsion is based on in vitro experiments (Autiero, De Smet et al. 2005).

Lessons from the cardiovascular system: While axon guidance to pelvic organs remains largely unexplored, information from other autonomic targets provides a framework. A well-studied case of autonomic axonal development relates to the innervation of the blood vessels and the heart. For an anatomical review refer to (Kawashima 2005). This is especially relevant given that it is thought that many sympathetic axons in tumours may be derived from those innervating the vasculature.

Succinctly, trophic factors such as netrin-1 were found to be essential for the sympathetic innervation of arteries, as shown in a transgenic mouse model (Brunet, Gordon et al. 2014). But arterial innervation only happens after birth, when axons sprout into target tissues and slowly innervate the arteries then forming neuroeffector junctions (Zheng, Felder et al. 1994, Brunet, Gordon et al. 2014).

Figure 1.4 shows a model for arterial innervation as described by Zheng, Felder et al. (1994).

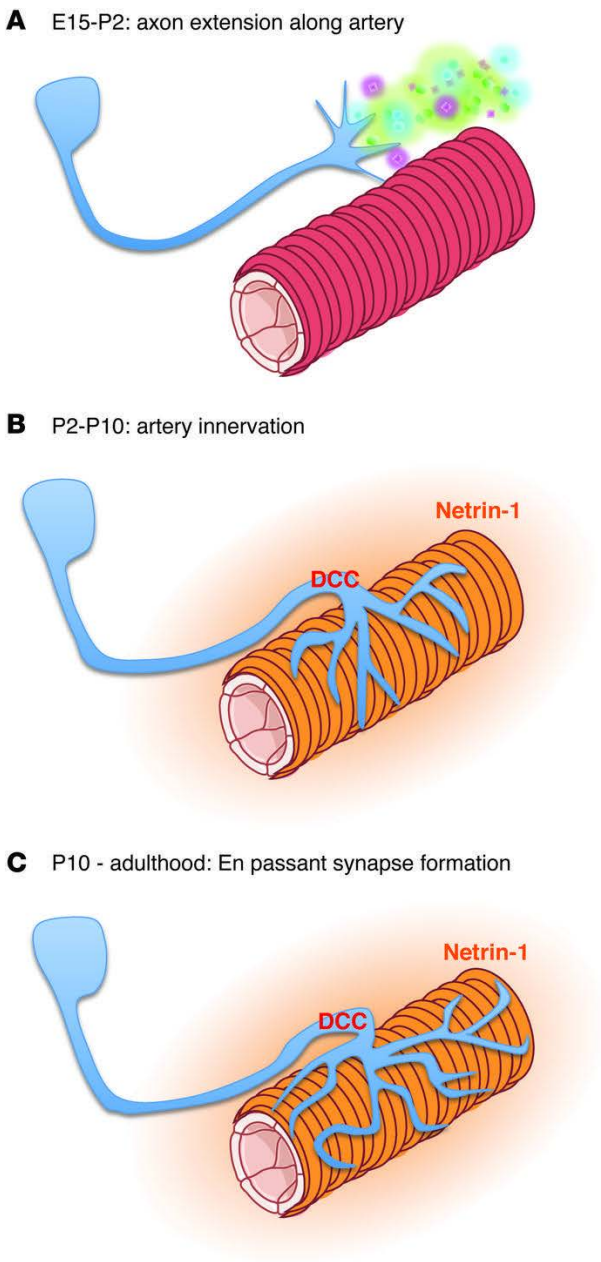


Figure 1.4 - Model for arterial innervation (Zheng, Felder et al. 1994). (A) Proximal axon extension along the blood vessel vasculature happens before birth and requires neurochemicals, such as endothelins, artemin, and neurotrophins, produced by the arteries (coloured dots). (B) Innervation of the vessels occurs at post-natal day 2, corresponding with the expression of netrin-1 by the smooth muscle cells in the arterial wall (orange). (C) Full blood vessel innervation and formation of synapses occurs around post-natal day 10.

1.4. Autonomic and sensory nervous systems in the normal ovary

The female reproductive tract is a complex collection of tissues coordinated by multiple endocrine and nervous signals (Jobling, O'Hara et al. 2014). A comprehensive review of the ovarian nerves and their role in the ovarian function was discussed over 10 years ago (Aguado 2002). Since then, little has been added to the origins, extrinsic and intrinsic distributions, density, and cytochemical characteristics of the ovarian neuronal innervation. It is likely that the reproductive systems nerves develop in close association with the innervation of the excretory system but, with a slight delay from this (Maggi 1993, Fowler, Griffiths et al. 2008, Said 2012, Katz 2013).

1.4.1. Gross neuro-anatomy of the ovary

The reproductive organs receive nerve fibres from three categories of peripheral nerves: 1) the sympathetic, 2) parasympathetic and 3) sensory. Sympathetic and parasympathetic nerves play a major role in the regulation of blood flow, secretions and motility of the hollow organs (uterus and vagina) (Jobling, O'Hara et al. 2014). The sensory nerves are responsible for transmitting sensory information such as stretch, pain and metabolic state of tissues to other structures of the nervous system (local reflexes), and ultimately to the spinal cord and the brain.

The innervation of the female reproductive system is anatomically similar between species in terms of the spinal origin of nervous signals and the types of neurotransmitters used (Burnett and Wesselmann 1999, Wesselmann 2001, Jobling 2011). Functional experiments in humans are difficult to perform, however reflex activation of the reproductive tract is conserved in a number of laboratory mammals

(Kulkarni, Wakade et al. 1976, Dees, Hiney et al. 1995, Gibbins, Matthew et al. 1996, Sato, Hotta et al. 1996, Hotta, Uchida et al. 1999, Jobling, Gibbins et al. 2004).

To understand the innervation of ovarian tumours it is useful to know the innervation of normal ovary.

Figure 1.5 represents what is described about the normal innervation of the female reproductive internal organs.

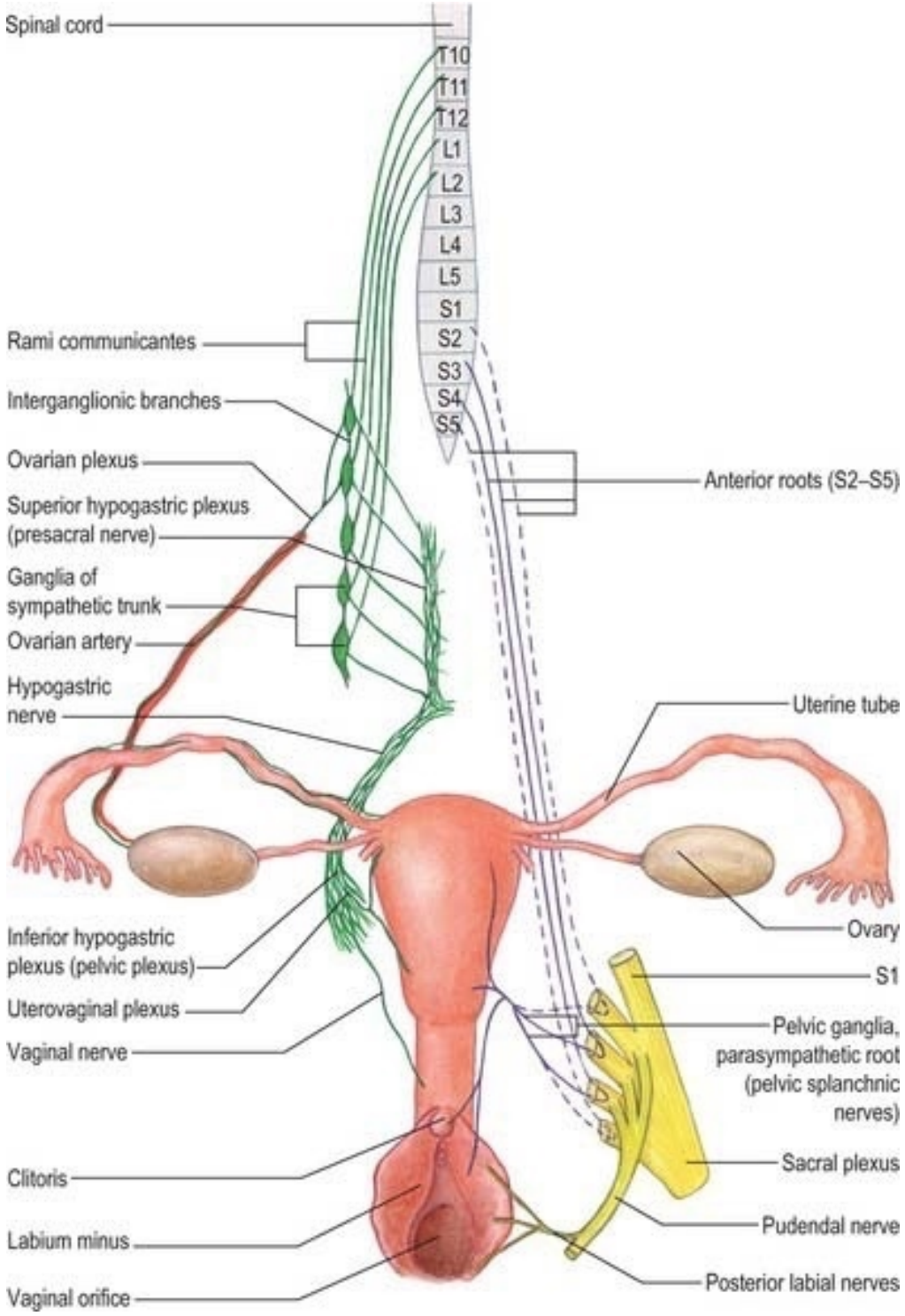


Figure 1.5 – Illustration of the nerve supply to the female reproductive tract. This shows the route the pelvic and pudendal nerves take as they feed off of the sacral plexus (Sobotta, Putz et al. 2006).

Fibres from the autonomic nervous system (visceral efferent fibres) and sensory fibres from the organs (visceral afferent fibres) concentrate in paired nervous structures designated as splanchnic nerves. These are named after the region of the spinal cord they arise from as: cervical, thoracic, pelvic, lumbar and sacral splanchnic nerves. The pelvic nerves carry parasympathetic nerve fibres. All the other splanchnic nerves are of sympathetic nature.

Nerve fibres extrinsically innervate the rat ovary by two paths: via the nerve in the suspensory ligament (superior ovarian nerve, SON), and via the ovarian plexus (OPN) along the ovarian artery (Malishevskaia and Brindak 1980) -

Figure 1.5. The somata of sympathetic axons that innervate the ovary are mainly housed in the suprarenal ganglia (SRG) and the T10 to L3 ganglia of the paravertebral sympathetic chain (Houdeau, Rousseau et al. 1998) – see Figure 1.5.

1.4.2. Anatomical evidence for innervation of the ovary

1.4.2.1. Origin of the ovarian nerve supply

External nerve supply: The major nerves supplying the ovary arise from the ovarian and hypogastric ganglia, which are associated with the aortic plexus adjacent to the junction between the aorta and the inferior mesenteric artery. These ganglia house the final autonomic motor neurons that project to the ovaries and upper uterus (Mahran, Fadel et al. 1971, Payer 1978, Houdeau, Rousseau et al. 1998, Beveridge, Johnson et al. 2016). These autonomic neurons receive input from the CNS via the splanchnic nerves. Also, the prehypogastric and ovarian ganglia comprise the aortic plexus that are bilaterally and symmetrically organized (Beveridge, Johnson et al. 2016). Some of the sympathetic innervation also arises from paravertebral chain ganglia (Klein and Burden

1988). In addition, axons from primary sensory neurons located in the dorsal root ganglia adjacent to the spinal cord segments T10-L1 travel to the ovary via the aortic plexus (Burden, Leonard et al. 1983, Chien, Li et al. 1991). In rodents, there is evidence of a vagal sensory innervation of the ovary (Burden, Leonard et al. 1983), but this is yet to be confirmed in other species (Chien, Li et al. 1991).

Nerve fibres extrinsically innervate the rat ovary via the nerve in the suspensory ligament (superior ovarian nerve, SON) and the ovarian plexus (OPN) along the ovarian artery. The SON include large adrenergic nerves, acetylcholinesterase-positive, embedded in the smooth muscle that expands from the celiac plexus and reaches the ovaries with the suspensory ligament (Lawrence and Burden 1980). It is mainly originated from neurons from the suprarenal ganglia (SRG) and the T10 to L3 ganglia of the paravertebral sympathetic chain (Houdeau, Rousseau et al. 1998). The OPN originates from the intermesenteric plexus in the ventral surface of the aorta, caudal to the superior mesenteric artery, and courses with the ovarian artery.

The nerve plexus that follows the ovarian vessels of the rat ovary have predominantly unmyelinated fibres and two types of neuronal cell bodies. One larger and like the autonomic postganglionic neurons and a second smaller with cytoplasmic granulated vesicles. Both synapse with the neuronal cell bodies and dendrites of nearby axons (Payer 1978).

Intrinsic neurons*:* Cells with the morphology of neurons were described in the medulla of the human ovary by Winterhalter in 1896 (as cited in (Tucker 2013)). Tyrosine hydroxylase- and low-affinity NGF receptor $p75^{NTR}$-immunoreactive neurons were found in the ovary of rhesus monkeys, their numbers decreasing with age. The same authors found neurons in the pig ovary (Dees, Hiney et al. 2006). No neurons

were observed in the ovaries of cats (Fink and Schofield 1970), whilst intrinsic neurons were found in Wistar rats and in fetoneonatal human ovaries (Anesetti, Lombide et al. 2001), but not in Sprague Dawley nor Long Evans rats (Fink and Schofield 1970, D'Albora, Lombide et al. 2000). This suggests species variation. Differences exist both in localization, number, morphology and biochemical phenotype of neurons (D'Albora and Barcia 1996, Anesetti, Lombide et al. 2001, D'Albora, Anesetti et al. 2002). Several ovarian intrinsic neuronal populations were shown to decrease remarkably with age in other primates, therefore associated to menopause and to reproductive function and competence (Anesetti, Lombide et al. 2001). Interestingly, the presence of these neurons in the ovary of Wistar rat, even though in small quantity when compared with primates, was correlated with longer reproductive life span in this rat strain (D'Albora, Lombide et al. 2000, D'Albora, Anesetti et al. 2002). Some of these neurons produce catecholamines – suggesting a role in steroidogenesis – some produce neuropeptides. Their number generally remains elevated throughout reproductive life until the menopause, when they decrease drastically (D'Albora, Lombide et al. 2000, Anesetti, Lombide et al. 2001). Their exact function remains unknown. However, it is held that intrinsic ovarian neurons are evolutionarily associated to the regulation of ovarian reproductive performance in higher species, which have a longer reproductive span (Anesetti, Lombide et al. 2001). This regulation includes, for example, control of ovarian luteal phase and ovulation, and steroid production (Erskine and Weaver 1988).

Intrinsic and extrinsic innervation assumed an anatomical complex association in higher primates in order to regulate ovarian function (LePere, Benoit et al. 1966, Dees, Hiney et al. 1995, Mayerhofer, Smith et al. 1998, Aguado 2002). The innervation of

gonads is found in all vertebrates and appear to be evolutionary conserved (Dees, Hiney et al. 1995, Anesetti, Lombide et al. 2001, Jobling, O'Hara et al. 2014).

1.4.3. Autonomic ovarian innervation

Adrenergic nerves: The sympathetic nerve supply to the ovary is well documented in most species (Stefenson, Owman et al. 1981). Most of the sympathetic innervation to the ovary is characterized by catecholamines and neuropeptide-Y (NPY) containing axons (McNeill and Burden 1986, McNeill and Burden 1987, McNeill and Burden 1987, Klein and Burden 1988). Much of the sympathetic supply goes to blood vessels and is vasoconstrictor (Jobling, Gibbins et al. 2003, Janig 2014). Sympathetic axons can also be found within the stroma and surrounding follicles, where it is associated with smooth muscle cells and the follicle wall (Walles, Groschel-Stewart et al. 1978).

Sympathetic nerve terminals are found along the vasculature, in the interstitial tissue and the ovarian follicles. Ovarian sympathetic nerve terminals can release several combinations of neurotransmitters. These include noradrenaline (NA), which gives the designation adrenergic to the nerves and neuropeptides such as neuropeptide Y (NPY), or vasoactive intestinal polypeptide (VIP). It should be noted that the overwhelming majority of data on specific subtypes of sensory (and autonomic motor) axons arise from animal studies. However, the peripheral nervous system is very highly conserved among all vertebrates in both their neurotransmitter profile and segmental origin (Jobling 2011, Jobling 2011).

Data from rodents show varicose adrenergic axons associated with follicles (Owman and Sjoberg 1966) and the interstitial gland (Stefenson, Owman et al. 1981, Burden, Lawrence et al. 1985). The interstitial gland is linked to the production of ovarian androgens, suggesting a link between the autonomic nervous system and androgen production (Curry, Lawrence et al. 1984, Curry, Lawrence et al. 1984). Adrenergic axons were detected adjacent to the follicles and the egg nests in guinea pig ovary as well as blood vessels (Papka, Cotton et al. 1985). These data suggest a role for autonomic innervation in follicle development and maturation (Burden 1972).

Cholinergic nerves: Anatomical evidence for a cholinergic innervation is confounded by the fact that reliable markers for cholinergic axons are relatively recent (Chen, Sharrad et al. 2015, Keast, Smith-Anttila et al. 2015). Early acetylcholinesterase labelling suggested a non-adrenergic innervation of the ovary but it is unclear if this may have included non-cholinergic sensory axons (Burden and Lawrence 1978, Lawrence and Burden 1980, Amenta, Cavallotti et al. 1981, Sporrong, Kannisto et al. 1985, Mayerhofer and Fritz 2002). More recently, Kozlowska reported immunoreactivity to the vesicular acetylcholine transporter (vAChT) in the ground plexus and isolated axons near follicles in porcine ovary (Kozlowska, Majewski et al. 2014). In humans, vAChT-IR axons were seen in the stroma and near blood vessels (Wojtkiewicz, Jana et al. 2014) but not near follicles. It is commonly assumed that parasympathetic innervation comes from postganglionic cholinergic neurons that reach the ovary via vagal efferents (Burden and Lawrence 1978) or branches of hypogastric plexus associated with OPN (Burden and Lawrence 1978, Burden, Leonard et al. 1983). However, the actual origin of these axons is not clear. While it is tempting to think that they are parasympathetic, presumably arising from the paracervical ganglia, they may

be sympathetic cholinergic axons. The female reproductive tract has a rich innervation of these axons, particularly in vasodilator neurons (Jobling, Gibbins et al. 2003, Gerendai 2004, Mowa and Papka 2004, Yuan, Gibbins et al. 2011, Monica Brauer and Smith 2015). And indeed, specific choline acetyltransferase (CHAT) antibodies were unable to identify cholinergic axons in the rhesus or rat ovaries (Mayerhofer and Fritz 2002).

While most of the female reproductive tract receives parasympathetic innervation, no substantial proof has been given of parasympathetic fibres in the ovaries (Papka, Cotton et al. 1985, Papka and Traurig 1988, Papka 1990, Traurig, Papka et al. 1991, Papka, McCurdy et al. 1995, Morris, Gibbins et al. 2005, Jobling and Lim 2008). Parasympathetic fibres are known to produce acetylcholine (ACh), which functions as a marker for the cholinergic nature for this type of nerves. Selective markers for cholinergic axons were developed relatively recently (e.g., antisera for vAChT or CHAT). Consequently, early reports using enzyme reactions for acetylcholinesterase probably mis-identified many adrenergic axons as cholinergic (Stefenson, Owman et al. 1981). Additionally reports of cholinergic axons along the oviducts of pregnant women may reflect "*en passant*" axons travelling to the uterus or cervix" (Kraus and Gombos 1990).

Therefore, it seems that ovarian innervation of mammals, including that of humans and primates in general, is predominantly (if not at all) of sympathetic and sensory nature (Stefenson, Owman et al. 1981, Klein and Burden 1988, Dees, Hiney et al. 1995, D'Albora, Lombide et al. 2000).

Sensory innervation: Ovarian sensory nerves containing substance P (SP), vasoactive intestinal polypeptide (VIP) and calcitonin gene related peptide (CGRP) are commonly reported in the literature (Dees, Ahmed et al. 1986, Calka, McDonald et al. 1988, Schultea, Dees et al. 1992). However, SP axons have seldom been detected in primates' ovaries (Sporrong, Kannisto et al. 1985, Kannisto, Ekblad et al. 1986, Schultea, Dees et al. 1992). Sensory neurons immunoreactive to galanin, pituitary adenylate cyclase activating polypeptide (PACAP) and neuronal nitric oxide synthase (nNOS) have also been observed (Majewski, Pidsudko et al. 1996, Majewski, Kaleczyc et al. 2002, Wojtkiewicz, Jana et al. 2014). Concomitantly, VIP-IR axons of sympathetic and sensory nature have also been described in rodents' ovary (Papka, Cotton et al. 1985, Ahmed, Dees et al. 1986).

Primary afferent sensory nerves innervate the female reproductive tract and follow the same pathways as the autonomic nerves. These fibres course through the autonomic ganglia and have their source somata in the thoracic and lumbosacral dorsal root ganglia (DRG) (Inyama, Wharton et al. 1986, Klein and Burden 1988, Papka 1990, Shew, Papka et al. 1990). Retrograde labelling from rat ovary confirmed the existence of neurons in DRG T12-L1 (Burden and Zary 2002) with the majority in T13 and L1, L2. Sensory fibres are immunoreactive to several peptide transmitters such as SP (Papka, Cotton et al. 1985), calcitonin gene-related peptide (CGRP) (Lara, McDonald et al. 1990) and vasoactive intestinal polypeptide (VIP). The neurotransmitters found in the ovary and their distribution pattern is summarized in Figure 1.6 and Table 1.2. Note that VIP is also a neurotransmitter in cholinergic vasodilator nerves as well as some sensory nerves (Papka, Traurig et al. 1987, Papka, Thompson et al. 1996, Jobling, Gibbins et al. 2003). The vagal system has also been implicated in the sensory

innervation of the female reproductive tract (Burden, Leonard et al. 1983, Dees, Ahmed et al. 1986, Papka, Collins et al. 1999) but it is equivocal whether this applies to the ovary (Inyama, Wharton et al. 1986).

Another molecule has also been found present and important in the ovarian cycle is nitric oxide (NO). Nitric oxide synthase (NOS) is found in sensory neurons but not in the autonomic motor neurons projecting to the ovary (paravertebral ganglia). A high percentage of sensory neurons that project to the ovary have NOS hence are capable of releasing NO (Jarrett, Price et al. 1994). The role of NO in ovarian function is not clear however it has been suggested that NO modulates ovarian steroidogenesis (Olson, Jones-Burton et al. 1996, Delgado, Casais et al. 2006). For a schematic of the postulated adrenergic and peptidergic innervation of the ovary see figure 1.6.

Finally it should be noted that for the female reproductive tract, innervation density and neuron excitability is strongly influenced by beta-oestradiol and to a lesser extent by progesterone (Papka and Mowa 2003). For example, pain threshold fluctuates with menstrual cycle and changes post menopause. Sympathetic nerves to the uterus retract during pregnancy under the influence of progesterone. Thus a women's reproductive status must be taken into any account when considering ovarian tumour innervation (Papka and Mowa 2003).

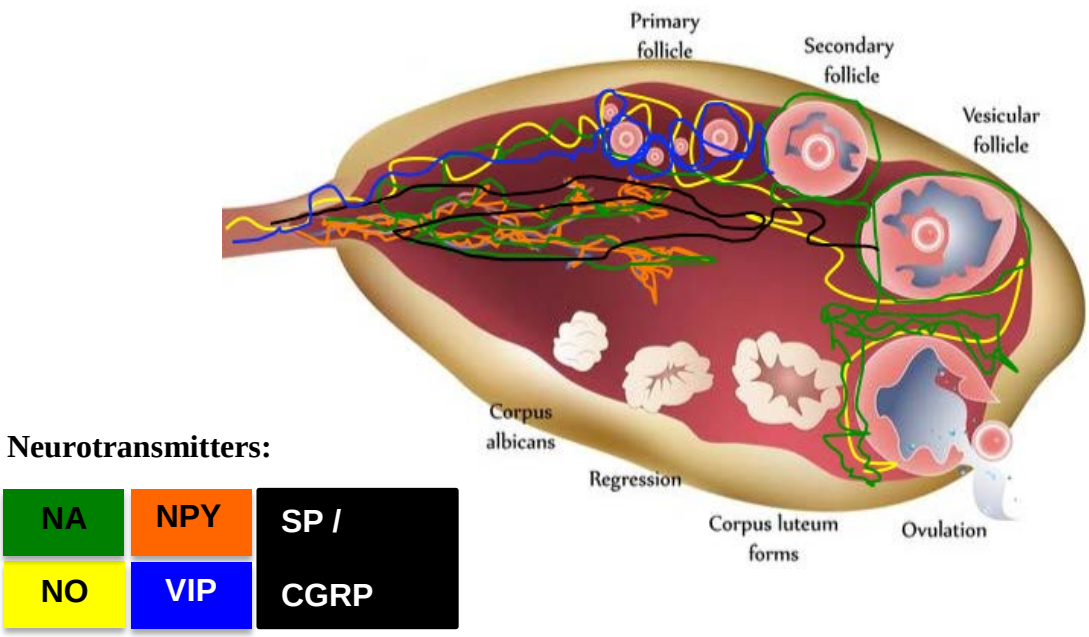


Figure 1.6 - Innervation of the normal ovary. Sympathetic and sensory axons arrive via the ovarian nerves. These nerve fibres release a variety of neurotransmitters, which can potentiate ovarian function.

1.4.4. Influence of autonomic nerves in ovarian function

Several histopathological studies have addressed the autonomic innervation of the ovary whilst not clarifying their role in ovarian function (Fink and Schofield 1971, Kannisto, Owman et al. 1984, Burden, Lawrence et al. 1985, Klein and Burden 1988, Lara, Dees et al. 1991, Malamed, Gibney et al. 1992, Doganay, Simsek et al. 2010, Uchida and Kagitani 2015). In addition, studying the innervation of the human ovary presents challenges on itself and important differences amongst species cannot be disregarded. Thus, human ovarian innervation remains controversial.

Ovarian innervation and blood flow: The ovary receives blood from the ovarian and uterine arteries. Blood flow has been linked to the ovulatory process of the rat

(Brannstrom, Zackrisson et al. 1998, Hazzard and Stouffer 2000, Zackrisson, Mikuni et al. 2000). Locally detected non-adrenergic/non-cholinergic neurotransmitters (NANC) neurotransmitters such as NO have been linked to ovarian blood flow regulation during the preovulatory period in the rat (Mitsube, Zackrisson et al. 2002). Considering the recognized role of the autonomic nervous system in regulating the circulatory system, a logical function of autonomic efferent nerves to the ovary is that of ovarian blood flow regulation and steroids secretion (Kawakami, Kubo et al. 1981, Hanada, Uchida et al. 2011, Morales-Ledesma, Vieyra et al. 2012, Trujillo, Morales et al. 2015, Uchida 2015). Sympathetic nerves to the ovary and NA have been shown to reduce blood flow to the ovaries by vasoconstriction of ovarian arterioles (from both OPN and SON) (Reynolds and Ford 1984, Uchida, Hotta et al. 2003, Stener-Victorin, Kobayashi et al. 2004, Hanada, Uchida et al. 2011). Furthermore, ovarian oestradiol production and ovarian blood flow seem to be controlled independently by sympathetic nerves (Uchida and Kagitani 2014, Uchida and Kagitani 2016). Of the two sympathetic pathways to the ovary (OPN and SON), stimulation of both reduces blood flow via alpha-1-receptors activation while only stimulation of SON reduces oestradiol production by activation of alpha-2-adrenoreceptors (Uchida, Hotta et al. 2003, Kagitani, Uchida et al. 2011). Interestingly, these sympathetic nerves and inhibition of ovarian oestradiol production can be activated by noxious cutaneous stimuli in rodents (Uchida, Hotta et al. 2003, Uchida, Kagitani et al. 2012). Therefore, the reflexive activation of the autonomic nervous efferents to the ovary may enclose an important adaptation of the female reproductive tract to internal and external changes; potentially to preserve fertility when environmental conditions were not prosperous for conception.

Ovarian innervation and folliculogenesis: It remains unclear at which developmental time the ovary becomes innervated. It is however suggested that it happens before folliculogenesis begins (Malamed, Gibney et al. 1992). Furthermore, early axons in the rat ovary are tyrosine hydroxylase-IR and limited to the medulla suggesting that pre-follicular events may happen in this region of the gland. This may also be physiologically significant as the rete ovary enters the ovary and the follicular formation starts in the medulla region (Byskov 1969, Byskov and Lintern-Moore 1973, Hirshfield 1991).

Denervation of the rabbit ovary had no effect on ovulation or pregnancy (Weiner, Wright et al. 1975). However, it affected the neuromuscular mechanism that controls ovulation through ovarian contractions (Weiner, Wright et al. 1977).

Denervation of gilts ovaries in the middle luteal phase of the oestrous cycle induced a drastic reduction on dopaminergic and TH nerve terminals that resulted in increased small follicles numbers whilst large follicles decreased (Jana, Dzienis et al. 2007). Thus, suggesting the importance of the peripheral nervous system in the control of the ovary.

Effects of electrical stimulation: Early studies showed electrical stimulation of the brain altered ovarian oestradiol and progesterone production while not affecting ovarian blood flow (Kawakami, Kubo et al. 1981, De Bortoli, Garraza et al. 1998, Morales-Ledesma, Vieyra et al. 2012). However, later investigations found that electrical stimulation of the distal portion of the SON and / or OPN lead to decreased ovarian blood flow and diameter of ovarian arterioles in the rat (Kagitani, Uchida et al. 2008, Hanada, Uchida et al. 2011, Kagitani, Uchida et al. 2011).

Stimulation of either the medial basal prechiasmatic area (PVA), ventromedial hypothalamus or areas of mesencephalon induced increased secretion of oestradiol E2 and progesterone in ovarian venous blood. At the same time, stimulation of the dorsal hippocampus, lateral amygdala and mesencephalic areas decreased these circulating steroids. Moreover, the cut of the ovarian nerves eliminated the production of oestradiol induced by PVA stimulation (Kawakami, Kubo et al. 1981). Therefore, the efferent neural system from the brain to the ovaries supplements the brain-pituitary-ovarian hormonal mechanisms in the regulation of ovarian steroid release, and consequently ovarian sensitivity to gonadotropins.

Moreover, it has been shown that SON, but not OPN stimulation, reduces oestradiol production (Kagitani, Uchida et al. 2008, Uchida, Kagitani et al. 2012). Conversely testosterone decreases in response to stimulation of either SON or OPN (Uchida and Kagitani 2014). Ovarian nerves have an inhibitory role in ovarian testosterone and blood flow via α1-adrenoceptors, while α2-adrenoceptors are activated when local oestradiol drops (Kagitani, Uchida et al. 2011). This indicates independent mechanisms of vasoconstriction and different steroids secretion in the ovary of the rat. The relationship between blood flow, steroids production and stimulation of ovarian nerves has been reviewed elsewhere (Uchida 2015, Uchida and Kagitani 2015). Correlated investigation to the human female gonad is yet to be performed.

So, the vasoconstriction of ovarian arterioles, and therefore of blood supply to the ovary, is regulated by the sympathetic innervation in the ovary. Correspondingly, the efferent neuronal system from the brain to the ovaries complements the hormonal feedback regulation of steroid production, sensitivity to gonadotropins and ovulation. Moreover, together with sympathetic nerves, sensory innervation regulates hormonal

secretion and this participation varies along the oestrous cycle (Trujillo, Morales et al. 2015).

Isolated studies have also shown that electrical stimulation of sympathetic nerves evokes contraction of isolated human Graafian follicles themselves (Owman, Sjöberg et al. 1975). Isolated Graafian follicles also contract in response to muscarinic agonists, but direct activation via cholinergic nerves has yet to be demonstrated (Walles, Edvinsson et al. 1976). Attempts have also been made to characterise the receptors and mechanism involved in the release of NA from the ovarian follicular nerves through electrical stimulation of contractile cells in the follicles theca externa (Kannisto, Owman et al. 1987). NA was suggested to stimulate progesterone release in bovine luteal cells (Battista and Condon 1986). NA also increases during the oestrous cycle in rats (Clausell and Soliman 1978). Although electrical and pharmacological stimulation of the follicles have an effect (Kannisto, Owman et al. 1987), the anatomical localization of NA releasing axons remains elusive.

Conceptually, ovarian function is rapidly regulated by responding to internal and external environmental changes thanks to the cooperation of central and autonomic innervation and ovarian hormones. The exact mechanism remains to be clarified.

A summary of the neurotransmitters reported in the ovary is depicted in Table 1.2.

Table 1.2 – Neurotransmitters reported in the ovary. The different neurotransmitters were categorised in "autonomic" , "sensory" and in "transmitters in intrinsic neurons", and their function and distribution in the ovary is noted.

	FUNCTION	DISTRIBUTION IN THE OVARY
Autonomic Neuronal Transmitters		
Noradrenaline (NA) / Norepinephrine (NE)	Regulation of ovarian steroidogenic sensitivity to catecholamines by regulating the number of functional β-adrenergic receptors (Aguado and Ojeda 1984)	Arise from pre- and paravertebral ganglia. Form a robust plexus that supply the vasculature, follicles and interstitial tissue (Schultea, Dees et al. 1992, Maggi 1993)
Acetylcholine (ACh)	Vascular tone. Ovarian function regulation (Fritz, Wessler et al. 2001)	Sparse innervation (Maggi 1993). Produced in granulosa cells of antral follicles (Fritz, Wessler et al. 2001, Mayerhofer and Fritz 2002)
Vasoactive intestinal peptide (VIP)	Vasodilation. Regulation of steroidogenesis regulation (Davoren and Hsueh 1985, Trzeciak, Ahmed et al. 1986). Ovarian differentiation (George and Ojeda 1987)	Species variability. Rare to moderate in human. Associated with blood vessels, follicles and interstitial tissue. Present in sympathetic and sensory nerves (Dees, Kozlowski et al. 1985, Papka, Cotton et al. 1985, Ahmed, Dees et al. 1986, Dees, Ahmed et al. 1986, Schultea, Dees et al. 1992, Maggi 1993)
Neuropeptide Y (NPY)	Vasoconstriction; involvement in ovarian steroidogenesis. LH production (Kalra and Crowley 1984, Crowley and Kalra 1987)	Moderate to rich innervation. Similar distribution pattern and density to NA fibres. Mostly perivascular, but also interstitial and associated with follicles (Mcdonald, Dees et al. 1987, Schultea, Dees et al. 1992, Maggi 1993)
Somatostatin	Modulation of ovarian function (Mcneill and Burden 1986). Inhibition of pituitary and ovarian hormonal responses (Prelevic, Wurzburger et al. 1990, Fulghesu, Lanzone et al. 1995). Regulation of ovarian steroidogenesis by mediating gonadotrophin and growth factor in the various ovarian cell types (Andreani, Lazzarin et al. 1995)	Present in ~ 37% of the sympathetic axons projecting to the ovary.

GABA (γ-aminobutyric acid)	Implicated in follicular wall contractions (Delrio and Caballero 1980, Kannisto, Owman et al. 1986, Erdo, Joo et al. 1989)	Source in the ovary is unknown. Detected in vacuole-like formations in the follicular fluid and oocytes (Delrio and Caballero 1980, Kannisto, Owman et al. 1986, Erdo, Joo et al. 1989)
Sensory Neuronal Transmitters		
Substance P (SP)	Vasodilator; nociception, facilitate vascular permeability (modulate NA transmission to the ovaries?); ovarian blood flow control (Dees, Kozlowski et al. 1985, Ojeda, Costa et al. 1985)	Perivascular, scattered in the stroma and around follicles, namely in the theca externa of antral follicles (Dees, Kozlowski et al. 1985, Papka, Cotton et al. 1985, Maggi 1993). Reaches ovary exclusively via OPN (Dees, Ahmed et al. 1986)
Calcitonin gene-related peptide (CGRP)	Vasodilator (Calka, McDonald et al. 1988, Gangula, Zhao et al. 1999); pain transmission; regeneration of nervous tissue; cardiovascular homeostasis and nociception.	More numerous than other peptide-containing axons, except for NPY. Prominent with vasculature, but also associated with follicles and interstitial tissue. Enter ovary via OPN (Calka, McDonald et al. 1988, Maggi 1993)
VIP	Sensory functions unknown	~ 20% of the afferent neurons (Maggi 1993)
Enkephalin (ENK)	Nociception; endogenous opioid (Facchinetti, Ruspa et al. 1986)	Found around arteries, between follicles and in the theca interna and granulosa cells (Cupo, Menezo et al. 1987, Maggi 1993)
Neuronal nitric oxide synthase 1 (nNOS)	Smooth muscle relaxation, vasodilator. Oocyte competence: ovulation, folliculogenesis, steroidogenesis and apoptosis (Shukovski and Tsafriri 1994, Jablonka-Shariff and Olson 1997)	In the stroma (Wojtkiewicz, Jana et al. 2014); granulosa and thecal cells (D'Albora, Anesetti et al. 2002)
Transmitters In Intrinsic Neurons In The Ovary		
ACh	Autocrine regulation: stimulates granulosa cells proliferation and synthesis of regulatory proteins of steroid production (StAR); increase sensitivity to LH	In granulosa cells (Fritz, Wessler et al. 2001, Mayerhofer and Fritz 2002)
NA / Dopamine	Neuroendocrine signals between oocytes and granulosa cells	In granulosa cells (Mayerhofer, Smith et al. 1998)

GABA, somatostatin, cholecystokinin	Function unknown in the ovary

Ovarian innervation and steroidogenesis: Neurotransmitters contained in the ovary are reported to stimulate steroidogenesis (Weiss, Dail et al. 1982, Delgado, Sosa et al. 2004, Orozco, Sosa et al. 2010, Bronzi, Orozco et al. 2011). Thus, the presence of neurotransmitters in the ovary suggests that ovarian innervation may be associated with ovarian development. VIP-IR axons can stimulate steroid release in the ovary of the immature rat. These axons seem to be of the first ones to populate the ovary (Advis, Ahmed et al. 1989). Sympathetic axons density increase, namely of VIP-IR axons, has been linked to early developmental changes in the ovary of rhesus monkeys and rats (Ahmed, Dees et al. 1986, Advis, Ahmed et al. 1989, Schultea, Dees et al. 1992). Furthermore, VIP-IR axons were targeting specific follicles indicating a VIP-dependent follicular selection at the early stages of the primate ovary development (Schultea, Dees et al. 1992).

Sectioning SON to the ovary does not affect blood flow, instead results in altered production of steroids throughout the oestrous cycle, in the rat (Aguado and Ojeda 1984). Similarly, denervation of gilts ovaries in the oestrous cycle induced both neuro-morphological and steroidogenic changes. Production of progesterone and testosterone amongst other steroids significantly decreased in follicles and plasma of denervated gilts (Jana, Dzienis et al. 2007). Hence, suggesting that nervous impulses to the ovary regulate gonadotropins production through ovarian steroids. In addition, differences exist in the ganglia supplying the innervation to the left and the right ovary. Their

selective ovariectomy have highlighted different capacities of each ovary to maintain normal hormone levels which varies during the oestrous cycle and depending in the integrity of the SON (Flores, Velasco et al. 2011). There is no evidence that this is linked to hypothalamic control. Thus, suggesting that the autonomic nervous system control the release of gonadotropins that regulate ovarian function.

1.5. Ovarian innervation and dysfunctions of the ovary

Neonatal exposure to androgens affects sympathetic innervation and was associated to the development of ovarian cysts in Wistar rats. Exposure to testosterone prevents ovulation and increases the density of noradrenergic nerves that contribute to the maintenance of polycystic ovaries. Sympathectomy does not rescue this effect; suggesting the relevance of androgens in the remodelling of ovarian sympathetic innervation and its association to the maintenance of ovarian cysts (Anesetti and Chavez-Genaro 2015). Significant remodelling of the cholinergic innervation of porcine ovaries submitted to chemical induction of cysts has shown to be dependent on the phase of the oestrous cycle, as well (Kozlowska, Majewski et al. 2014). Congruently, changes in the cholinergic and sensory innervation of adult women with polycystic ovaries have also been described (Wojtkiewicz, Jana et al. 2014). However, the nervous connection has not been investigated in ovarian tumours.

1.6. Growth factors and ovarian function

Growth factors also interact with the ovarian hormonal cycle. For a comprehensive review see Dissen et al, 2002 (80). Neonatal rats treated with NGF antibody showed follicular growth arrest and reduced production of androgens and oestradiol (66). During the initial surge of gonatropins at pubescence the TrkA (tyrosine receptor kinase A) gene (receptor for NGF) is expressed in periovulatory follicles. Simultaneously, there is a specific and rapid increase of 100-fold in TrkA messenger RNA (mRNA). Various mRNAs encoding tyrosine hydroxylase receptor for neurotrophin-4/5 (Neurotrophin receptor 4/5, NT-4/5), brain-derived neurotrophic factor (BDNF), and NT-3 (Neurotrophin receptor 3) were consistently low throughout growth and development. NGF mRNA levels in the interstitial cells increase at proestrous, peaking at ovulation. Interleukin-1 beta (IL-1β) is a known regulator of LH levels; this increase both the expression of TrkA and NGF in ovarian cells. IL-1β also promotes prostaglandin E2 (PGE2) release, and it has been shown that NGF antibodies and TrkA receptor blockers can inhibit this effect. Thus, it can be postulated that the synergistic actions of TrkA and NGF may have the potential to control follicular growth (81). Another NGF receptor, $p75^{NTR}$, has also been found in nerves innervating the ovarian vasculature, interstitial tissue and developing follicles.

NGF and its receptor TrkA are known to be present in the granular cells (GC) of human preantral and antral follicles, as well as in theca cells of antral follicles. Increased NGF expression induce the secretion of oestradiol E(2) and reduce the progesterone release, in the human GC (Salas, Julio-Pieper et al. 2006). Moreover, NGF is also known to induce the expression of functional FSH receptors (FSHR) in preantral rat follicles (Salas, Julio-Pieper et al. 2006). Therefore, NGF potentiates the

increase secretion of E(2) in preovulatory human ovary while preventing early luteinisation via progesterone secretion inhibition (Salas, Julio-Pieper et al. 2006).

Other neurotrophic factors, such as G-CSF (granulocyte colony-stimulating factor), are known to affect tumour progression by supporting the outgrowth of cholinergic nerves in prostate tumours (Dobrenis, Gauthier et al. 2015). Thus, suggesting a relationship between nerve growth factors and tumour development. This also suggests that cytokines, like G-CSF, may promote tumour progression by a combination of neurogenic and hematopoietic activities that collaborate in the tumour microenvironment (G-CSF promotes the infiltration of myeloid cells in the tumour microenvironment, leading to an immunosuppressive response and tumour progression) (Dobrenis, Gauthier et al. 2015). It is, therefore, likely that similar synergistic interactions exist in ovarian cancer development. Deciphering such interactions will allow us to develop better cancer treatments and improve patient survival and life quality.

1.7. Cancer

Cancer defines a group of diseases that involve abnormal cell growth. When metastatic its main characteristic is the potential to spread, and invade other tissues and organs. It is currently one of the most common diseases worldwide and its rate tends to increase as the aging population and sedentary lifestyles also rise. Immunosenescence and DNA replication and methylation errors are currently believed to be the main culprits for the growing number of cancer cases (ThyagaRajan and Felten 2002, Shilpa, Bhagat et al. 2014). However, the rising cases of child and young adult cancer reinforce

the concept that a number of abnormal cell signalling cascades and environmental factors also contribute to cancer progression. This coupled with the unchanged survival rates for many cancers underscores the urgent need for alternative treatment options (AIHW 2012, AIHW 2012).

1.7.1. Ovarian cancer types

Ovarian cancer is a common gynaecologic cancer in developed countries. Over 3-17 per 100,000 women aged 40 or less years old will develop ovarian cancer. An obvious challenge for this particular age group is to balance the required treatment while preserving reproductive potential (Ng, Low et al. 2012). Moreover, over half of the women diagnosed with ovarian cancer will die from the disease. Despite the biomedical advancements, this poor survival rate has been constant, worldwide, for over 30 years. Robust methods of early detection and new treatment options are urgent (Jones and Drapkin 2013).

Ovarian cancer epidemiology and subtypes have been extensively analysed and reviewed (Lengyel 2010, Stack 2010, Ng, Low et al. 2012, Prat 2012, Coward, Middleton et al. 2015). Briefly, ovarian tumours can sprout from three different cell types: epithelial, germ, and sex cord stromal cells. Epithelial ovarian tumours account for ≥90% of all the ovarian cancers.

Considering the genetic stability, the tumours are further divided in two types: Type I tumours or low-grade develop slowly and progressively from well-organised precursors (mostly, borderline tumours); Type II or high-grade tumours progress

rapidly and are genetically unstable – harbour p53 mutations and extensive DNA copy-number alterations (Ng, Low et al. 2012, Jones and Drapkin 2013).

Based on histopathology and molecular genetic alterations, ovarian cancer cases commonly divide into: high-grade serous (~70%), endometrioid (~10%), clear-cell (~10%), mucinous (~3%), and low-grade serous carcinomas (< 5%). These account for over 95% of ovarian cancer cases (Prat 2012) – see Figure 1.7. Table 1.3 addresses the histology of ovarian cancer subtypes.

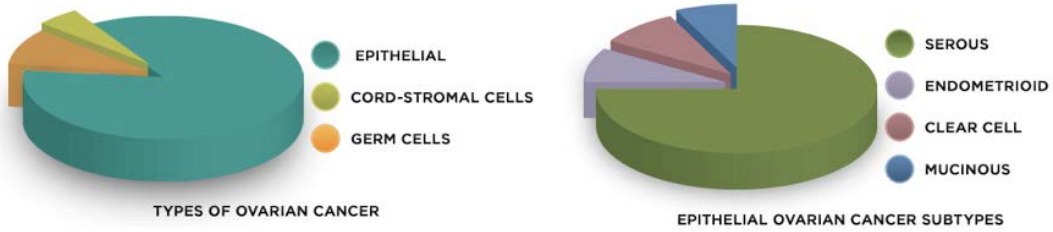


Figure 1.7 – Types of ovarian cancer and respective global proportions. Image from http://www.clearityfoundation.org/about-ovarian-cancer/ovarian-cancer-basics.aspx. Accessed December 2014.

Table 1.3 - Putative precursors for the different types of ovarian cancer. GI = Gastrointestinal tract.

Types of differentiation	Tissue that tumour most closely resembles
Serous	Fallopian tube epithelium
Mucinous	GI tract or endocervical epithelium
Endometrioid	Proliferative endometrium
Clear cell	Gestational endometrium
Transitional cell (Brenner)	Urinary tract epithelium

The genetic profile also contributes for the classification of the ovarian tumours (Rosen, Yang et al. 2009, Lengyel 2010). This is linked to cancer aggressiveness. Data from other solid tumours suggest that some genes may be important for growth of new axons (axonogenesis) (Ayala, Dai et al. 2008, Albo, Akay et al. 2011, Raju and Ibrahim 2011, Magnon, Hall et al. 2013, Goehrig, Detjen et al. 2014, Zhao, Yang et al. 2014). However, whether this applies to ovarian tumours is unknown and forms part of this project.

Table 1.4 is adapted from Prat, 2012 (Prat 2012) and Nik et al., 2014 (Nik, Vang et al. 2014) and summarizes the several categories of epithelial ovarian cancer and associated histopathological and genetic pattern.

*Table 1.4 - Clinical and molecular features of the most common types of ovarian cancer (epithelial). * Hereditary nonpolyposis colorectal carcinoma. HG = High-grade; SC: serous carcinoma; LG= Low-grade; SC: serous carcinoma; MC: Mucinous carcinoma; EC: Endometrioid carcinoma; CCC: Clear-cell carcinoma. Adapted from (Prat 2012, Nik, Vang et al. 2014).*

	Risk factors	Precursor lesions	Pattern of spread	Molecular abnormalities	Chemosensitivity	Prognosis
TYPE I TUMOURS						
LGSC	?	Serous borderline tumour	Transcoelomic spread	BRAF, KRAS, ERBB2	Intermediate	Intermediate
LGEC	HNPCC *	Atypical endometriosis	Usually confined to pelvis	CTNNB1 PIK3CA PTEN ARID1A	High	Favourable
MC	?	Cystaednoma / Mucinous borderline tumour	Usually confined to ovary	KRAS HER2	Low	Favourable
CCC	?	Atypical endometriosis	Usually confined to pelvis	PIK3CA ARIDIA FBXW7	Low	Intermediate
TYPE II TUMOURS						
HGSC	BRCA1 BRCA2	Fallopian tube epithelium	Very early transcoelomic spread	BRCA p53	High	Poor
HGEC	?	Not recognized	?	p53	?	?
Undifferentiated	?	Not recognized	?	Unknown	?	?
Carcinosarcoma	?	Not recognized	?	p53	?	?

1.8. Tumour microenvironment

The tumour microenvironment designates the cellular environment that surrounds the malignant tumour cells and includes blood vessels, immune cells, fibroblasts, extracellular matrix (or/and stroma) and other molecules, enzymes and factors.

The 'crosstalk' between the different cells and secreted molecules surrounding the tumour results in complex signalling pathways that influence tumour growth. A representation of the various processes involved in tumour development is summarized in Figure 1.8.

Briefly, rapid cell division is known to induce changes in surrounding tissue to accommodate the increased metabolism of cell division and the nutrients required for new cells. Usually this is a positive response, as in the cascade of events that occur during wound healing including the stimulation of new blood vessels, angiogenesis. However, solid tumours also alter their microenvironment in order to maximize their proliferation. This notion is better known through tumour angiogenesis, where tumours induce the growth of new blood vessels to increase their oxygen and nutrients supply. This usually increases the likelihood of metastases. Furthermore, each type of tumour appears to respond to cancer cell secretions through different signalling pathways, some of those working synergistically (Davidson, Reich et al. 2003, Roato, Grano et al. 2005, Cole and Sood 2012).

Typically, the tumour microenvironment is poor in oxygen, which leads to the activation of hypoxia-inducible factors that regulate gene expression (Voss, Niggemann et al. 2010). Under low levels of oxygen tumour cells release substances such as growth factors and cytokines that induce their vascularization (angiogenesis), lymph vessel

development (lymphangiogenesis) and, as will be outlined below, innervation (neoneurogenesis or axonogenesis). These leads to metabolic changes, including a reduction of respiration that end up affecting the proliferation, differentiation, resistance to apoptosis and metastatic potential of tumour cells. For instance, human prostate cancer cells PC-3 are known to increase their migratory potential under hypoxic conditions (Voss, Niggemann et al. 2010). On the other hand, metformin, a common anti-hyperglycaemic drug, inhibits the development and metastasis of ovarian cancer cells by reducing cell-extracellular matrix interactions, neovascularization and macrophage infiltration (Wu, Li et al. 2012).

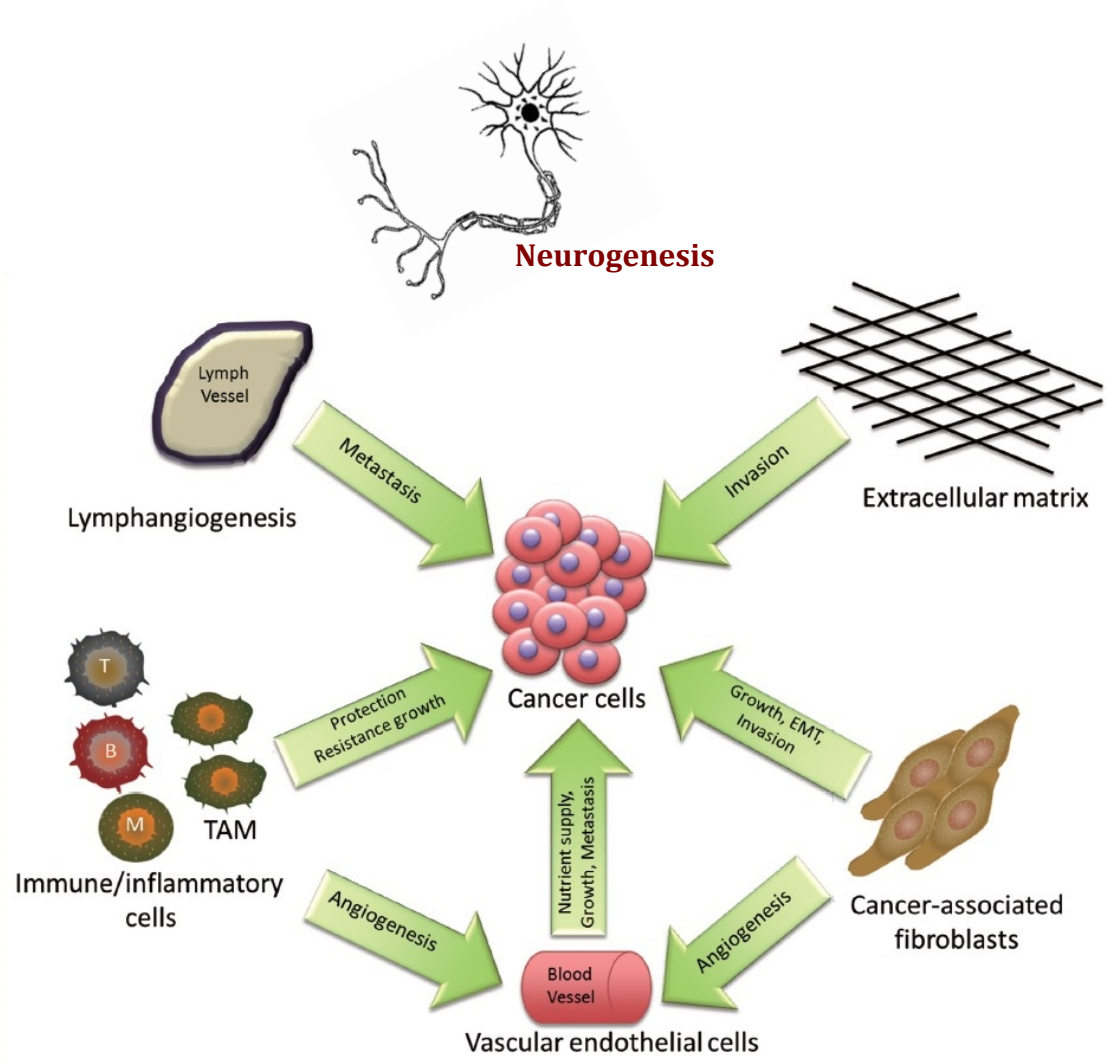


Figure 1.8 - The tumour microenvironment in tumour development. Cancer associated fibroblasts, tumour endothelial cells and tumour-associated macrophages express several molecules such as angiogenic promoting factors, growth factors and matrix-degrading enzymes that facilitate tumour proliferation and metastasis. (Adapted from Leyva-Illades, McMillin et al. (2012))

1.8.1. Neurons as part of the tumour microenvironment

Neurotransmitters have been shown to increase cancer cells migratory activity and to correlate with the suppression of cancer immune response – the reciprocal interactions between cancer cells and nerves, neurotransmitters and neuropeptides (Chedotal, Kerjan et al. 2005, Palmer, Sant Cassia et al. 2007, Shi, Liu et al. 2013). Also, their correlation to the cancer prognosis have been previously discussed (Mancino, Ametller et al. 2011).

Furthermore, the presence of nerve endings in tumour tissue has been described in several cancers, such as prostate and breast (see below - subheading 1.8.2.2).

Axon guidance molecules (e.g. slits and netrins) have also been described to be widely expressed in several cancers with possible roles in their microenvironment. These molecules are also known to regulate vascularization, cell migration and apoptosis in normal and cancerous tissues (Chedotal, Kerjan et al. 2005, Mehlen, Delloye-Bourgeois et al. 2011).

1.8.2. Neurotransmitters and cancer

Endogenous chemical messengers are generally designated by neurotransmitters. They are synthesized in the neurons and released during synapses, transmitting messages between a receptor and a target cell, through binding to specific receptors. Typically, neurotransmitters are divided into three categories: aminoacid amines (glutamate, glycine, GABA), biogenic amines (dopamine, norepinephrine, epinephrine and serotonin), and neuropeptides (NPY, VIP, opioids, etc.). The latter group keeps on growing with new identification of new neuroactive substances with identical physiological effects to neurotransmitters.

Several studies have described the influence of neurotransmitters in the immune system (Qiu, Peng et al. 1995, Qiu, Peng et al. 1996, Basu and Dasgupta 2000, Pacheco, Riquelme et al. 2010, Carreno, Gonzalez et al. 2011, Rosas-Ballina, Olofsson et al. 2011, Wong, Jenne et al. 2011) and in tumour cells (Lang and Bastian 2007, Jobling, Pundavela et al. 2015).

A tumour in itself is not a self-independent unit and it needs to interact with its surroundings, including the neuroendocrine system, in order to remain and progress. Tumour cells are known to express receptors for several neurotransmitters (Kondratenko, Zacharova et al. 1995, Lang and Bastian 2007, Schuller and Al-Wadei 2010, Vilardi, Bravo-Calderon et al. 2013, Kissick, On et al. 2015). Therefore, neurotransmitters released by the autonomic or the peripheral nervous system may react with specific receptors in tumour cells. Further helping their establishment. Concomitantly, tumour cells can also produce and release neurotransmitters (Lang and Bastian 2007, Li, Sun et al. 2013, Jobling, Pundavela et al. 2015). How this is regulated remains uncertain, but some hypothesize an autocrine/paracrine loop in the tumour microenvironment. In fact, there is a growing body of evidence suggesting that neurotransmitters and the nervous system are in fact essential for the development and progression (including migration, invasion and metastasis) of many cancers (Lang and Bastian 2007, Li, Sun et al. 2013).

The importance of neoangiogenesis and neolymphangiogenesis as hallmarks of cancer development has been established (Hanahan and Weinberg 2011). Recently, neoneurogenesis, the growth of new axons, is receiving more interest. This formation of new axons requires the interplay of neurotrophic growth factors, extracellular matrix

and neurotransmitters released by the growing axon (Hagg 2009). Interestingly, many of the cues that drive the formation of new blood vessels also seem to drive axon growth. For example angiogenesis promoting factors like VEGF can also promote neurogenesis (Lazarovici, Marcinkiewicz et al. 2006).

Several clinical descriptions have implicated nerves in the progression and outcome of oesophageal, cardiac and prostate cancers (Blackshaw and Dent 1997, Partosoedarso and Blackshaw 1997, Lu, Zhou et al. 2003, Page, Slattery et al. 2005, Ayala, Dai et al. 2008, Gupta, Varghese et al. 2009, Magnon, Hall et al. 2013).

1.8.2.1. Cancer Pain

While the ability of substances released from nerves to promote cancer progression has obvious implications for chemotherapy, the ability of substances released from cancer cells to alter nerve function has implications for cancer pain. Some cancers are extremely painful (pancreatic, and bone cancer) whereas others including ovarian and breast cancer are relatively pain free. Whether this is a consequence of the relative proportions of autonomic versus sensory axons invading the tumours is unknown. Although my thesis will not specifically address pain it is worth noting that anti-NGF based pain therapies have been proposed as possible adjuvant therapies for some cancers (Jobling, Pundavela et al. 2015).

1.8.2.2. Nerves in prostate cancer

An investigation on the prostate ultrastructure over thirty years ago suggested that tumours spread through perineural spaces (Hassan and Maksem 1980). Although perineural invasion has been known for some time, the concept that nerves could invade tumours has only recently been accepted. Recently, Magnon and co-workers (Magnon,

Hall et al. 2013) in a landmark paper highlighted the contribution of nerves to the prostate tumour microenvironment. Using human prostate biopsies, they showed that high density of nerve fibres around tumours correlate with poorer prognosis. To understand the mechanisms, they used mouse models of prostate cancer. Surgical or chemical destruction of sympathetic nerves prevented the growth of tumours in early-stages; and chemical inhibition of parasympathetic nerves inhibited tumour proliferation. This suggests that targeting the autonomic nervous system may prove efficacious in prostate cancer. The cues that drive nerves into tumours are still being worked out, however, our laboratory has shown that expression of proNGF is positively correlated with Gleason score – which is linked to poor prognosis. Furthermore *in vitro* experiments suggest that proNGF released by cancer cells is sufficient to drive axon growth (Pundavela, Demont et al. 2014).

1.8.2.3. Nerves in breast cancer

Recent reports support the presence of axons in breast tumours. Innervation has been associated with tumour grade, aggressiveness and patient survival showing an important role for nerves in therapeutic guidance of cancer patients (Zhao, Yang et al. 2014). The mechanisms by which nerves influence breast tumours remain to be fully elucidated. However, our laboratory has data that strongly implicates NGF (Pundavela, Roselli et al. 2015). This growth factors supports neuron growth and survival through interaction with their receptors TrkA and $p75^{NTR}$; in contrast, the latter potentiate apoptotic responses when binding with the receptor sortilin. Recent findings have shown that the distribution of TrkA and $p75^{NTR}$ is not limited to the nervous system and offer new targets against both breast cancer (Davidson, Reich et al. 2004, Lagadec, Meignan et al. 2009, Pundavela, Roselli et al. 2015) and prostate cancer (Gallick, Corn

et al. 2012, Ojemuyiwa, Madan et al. 2014, Pundavela, Demont et al. 2014, Jobling, Pundavela et al. 2015), as shown by our laboratory.

The above data from our laboratory and others suggest that nerves contribute to tumour progression for breast and prostate cancer. Recent data from a mouse model of gastric cancer also strongly link nerves with tumour progression (Zhao, Hayakawa et al. 2014). Combined, these studies imply that all solid tumours may attract and interact with peripheral nerves. A major aim of this project is to test this hypothesis in another class of solid tumours, ovarian cancer.

1.9. The Cervix

The cervix consists of the lower part of the uterus connecting the vagina with the main uterine body. It is a different tissue, with characteristic and distinct anatomy and histological features from the uterus. The collagen-rich cervix acts as a mechanical sphincter during pregnancy and labour. The remarkable flexibility of the uterus and the cervical opening have been extensively studied for decades.

1.9.1. Anatomy of the cervix

The adult, non-pregnant cervix is narrower and more cylindrical than the body of the uterus and contains large, branched tubular glands. It is affected by the menstrual cycle and by ovarian hormonal secretions, which stimulate functional changes to create a more prosperous environment for spermatozoa transport within the cervical canal. Furthermore, epithelial changes occur with aging (Ferenczy 1982, Ross, Kaye et al. 2002). The cervix measures about 2 to 3mm in thickness can be divided in to two

regions: the ectocervix and the endocervical canal or endocervix. The ectocervix projects to the vagina and is made of stratified squamous non-keratinized epithelium. It has an opening that marks the transition to the endocervical canal, named external *os* or *ostium* of the uterus. The endocervix is lined with mucus-secreting simple columnar epithelium and, ends in a narrowing designated internal *os* or isthmus where the uterus begins (Ferenczy 1982, More 1984, Ross, Kaye et al. 2002). Between ectocervix and endocervix there is an abrupt transformation zone with the squamous and columnar epitheliums. The transformation zone is in the cervical canal before puberty and after menopause but, just outside the external *os* during reproductive age. Metaplastic changes or lesions on this zone often constitute pre-cancerous diagnostics. The cervical epithelial cells are constantly exfoliating into the vagina allowing early detection of lesions through simple cytological analysis of Papanicolaou smears (Ross, Kaye et al. 2002). Comprehensive descriptions of the cervical anatomy have been published (Ferenczy 1977) – see also Figure 1.9.

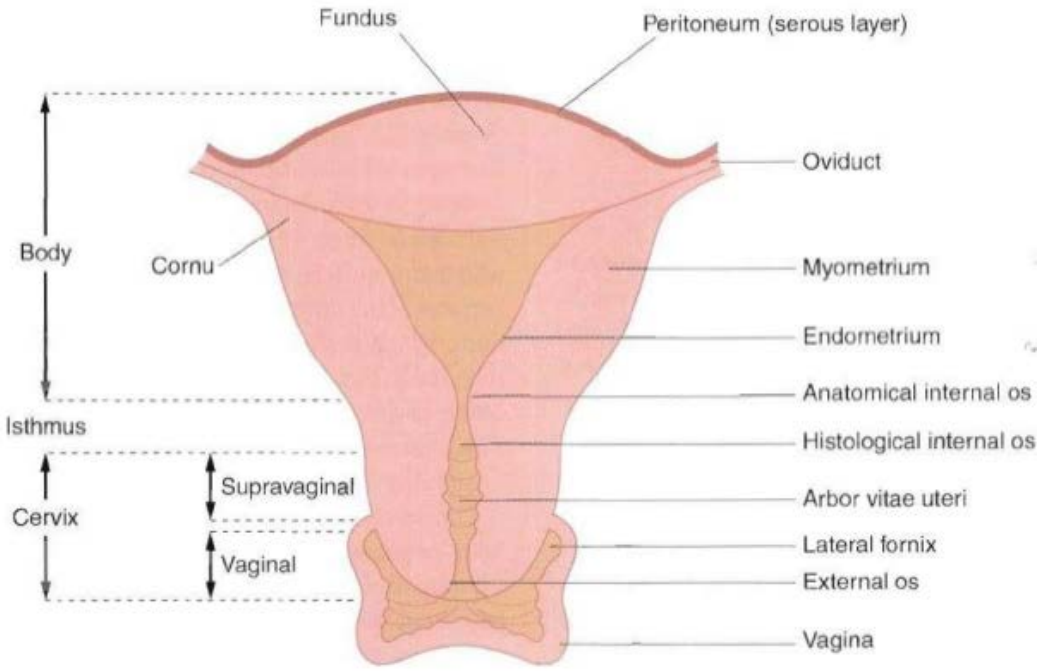


Figure 1.9 – Anatomy of the cervix. The ureter runs about 1 cm laterally to the supravaginal cervix and, the cervix-vaginal portion projects into the vagina to form the fornices. The upper part of the cervix is mainly involuntary smooth muscle while the lower cervix is fibrous connective tissue. The mucous membrane of the endocervix devises posterior and anterior columns that fold out forming the arbor vitae. Abundant glandular cells secrete a clear alkaline mucus that makes the vaginal discharge. The endocervical epithelium is cylindrical and, ciliated in the upper two thirds and then transforms into stratified squamous epithelium close to the external os. This squamous-columnar junction region is designated transformation zone and its cells are rapidly dividing. The great majority of cervical carcinomas arise from this area. From http://www.slideshare.net/yapa87/hypertrophic-elongated-cervix Accessed 17 Nov. 16.

1.9.2. Functions

The cervix has two main functions. It is responsible for facilitating the passage of sperm into the uterine cavity through dilation of the external and internal *os*. Plus, it maintains the upper female reproductive tract sterile, protecting the uterus and upper tissues from bacterial invasion. The monthly shedding of the endometrium, thick cervical mucus and narrow external *os* are responsible for maintain this environment sterile.

1.9.3. Innervation of the cervix

The uterus, uterine tube, cervix and upper vagina are manly innervated by branches of the intermediate and inferior hypogastric plexus. The sympathetic nerves originate in the lower thoracic segments of the spinal cord and pass through the lumbar splanchnic and the inferior mesenteric / hypogastric plexuses to the end target of the uterovaginal plexus. The parasympathetic nerves come out of the sacral levels 2 to 4 and pass through the pelvic splanchnic to the inferior hypogastric plexus in to the uterovaginal plexus. Note that the pudendal nerve (S2-S4) carries no parasympathetic nerve fibres. Visceral afferent nerve fibres from the fundus and body of the uterus travel retrograde with the sympathetic nerves along the hypogastric and superior hypogastric plexus nerves to the lower thoracic segments of the spinal cord. Afferent nerve fibres from the upper vagina and cervix travel retrograde along the pelvic splanchnic nerves (S2-S4) and on the pudendal nerve.

1.10. Cervical Cancer

Cancer of the cervix originates from the mucosa of the surface of the cervix or from the cervical canal. Carcinoma of the uterine cervix grows locally and may spread in continuity to the uterus and paracervical tissues, and pelvic organs.

1.10.1. Epidemiology and Aetiology

Cancer of the cervix is the fourth most common female disease and the second most common cancer in the female reproductive tract, in both incidence and mortality. About 530,000 new cases and 265,000 related-deaths are registered annually (Kurman, Carcangiu et al. 2014, Bermudez, Bhatla et al. 2015).

It is the second most diagnosed cancer and third most common cause of cancer related death amongst females in developing countries. Over 85% of new cases are diagnosed in economically disadvantaged people. About 90% of cervical cancer deaths occur in low-resource regions of the world (Bermudez, Bhatla et al. 2015).

The incidence of cervical cancer has markedly declined in many developed countries, mainly due to cytological screening programmes (Kurman, Carcangiu et al. 2014). The infection of female genitalia with human papilloma virus (HPV) has been strongly associated to the origin of cervical cancers (Gravitt, Kovacic et al. 2007, Schiffman, Castle et al. 2007, Castle, Gravitt et al. 2008). Thus, vaccination programs for HPV and co-essentially against cervical cancer have been installed in numerous developed countries (Franco, Cuzick et al. 2006, Muresan 2008, AIHW 2012, Kurman, Carcangiu et al. 2014). Nonetheless, in Australia alone over 900 new cases of cervical cancer are expected to be diagnosed and 250 women died from the disease in 2016. The 3-5 year survival rate has remained at about 70% since the late 1960s (AIHW 2012).

1.10.2. Cervical cancer onset

Most cervical tumours begin in the squamocolumnar junction (SCJ) lining of the cervix - Figure 1.10. Initially normal cervical cells undergo pre-cancerous changes that eventually will lead to invasive cancer if left untreated. Several terms are used to describe these pre-cancerous conditions: cervical intraepithelial neoplasia, squamous intraepithelial lesion, and dysplasia. These changes are usually identified through a cytological exfoliative screening test as the Papanicolaou (pap) smear test, and treated immediately to prevent the cancer from establishing (Franco, Cuzick et al. 2006, Schiffman, Castle et al. 2007).

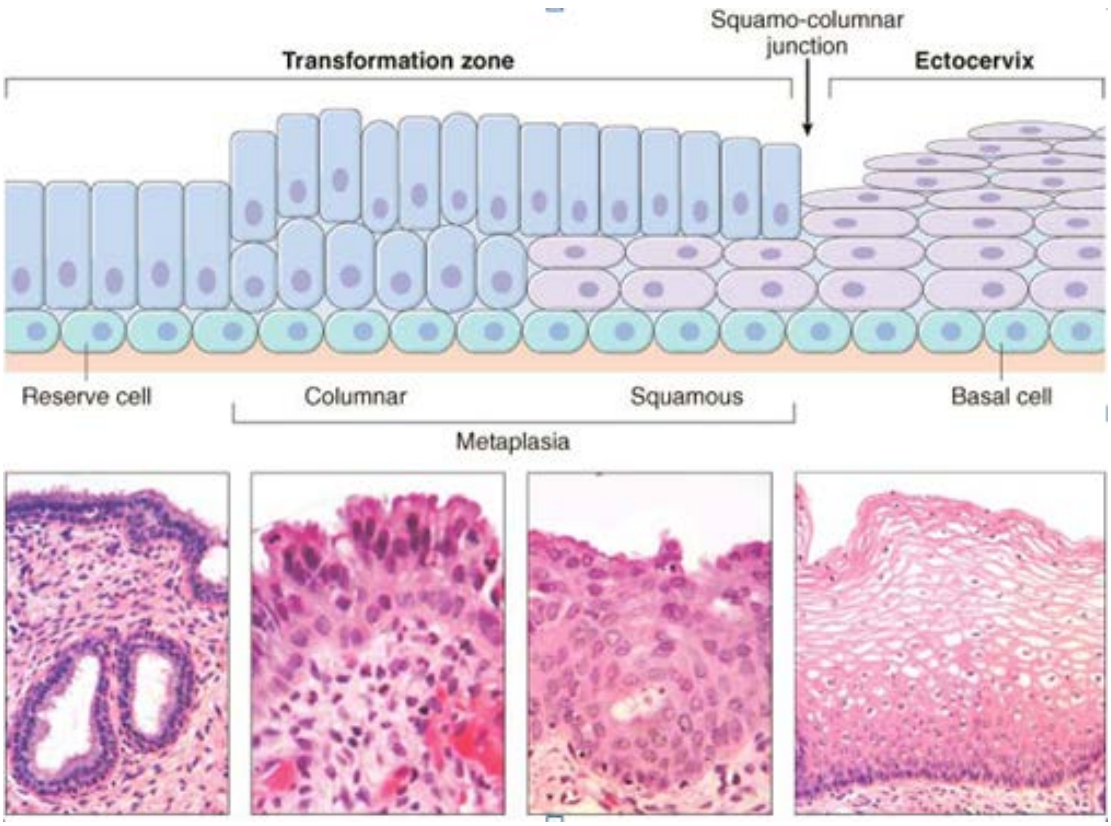


Figure 1.10 – Histology of the SCJ and transformation zone. http://www.slideshare.net/lluketic1/cervical-carcinoma-presentation. Accessed December 2014.

Squamous cell carcinomas account for almost all cervical cancers, and consist of cancerous masses in the epithelial lining of the ectocervix. While the other about 10% of the tumours are usually adenocarcinomas which consist of cancer of the cells found in the lining of the cervix. A rare number of mixed cell cervical (or adenosquamous) carcinomas and neuroendocrine cell carcinomas have also been described (~5%).

Similarly, to ovarian cancer, cervical cancer is asymptomatic in the early stages or symptoms confused with other common ailments. These symptoms include vaginal discomfort or discharge, urinary problems and inter menstrual bleeding. In late stages, the cancer can induce painless haematuria, chronic urinary frequency, altered bowel movement, leg oedema, pain and hydronephrosis, and generalised pelvic discomfort or pain.

1.11. Rationale And Overarching Hypothesis

Evidence for axon invasion into solid tumours, especially those of the breast and prostate is mounting. In these hormone-sensitive cancers, axon infiltration is correlated with worse prognosis. Evidence from our laboratory suggests that axon infiltration is partly driven by the release of neurotrophins by cancer cells which attract axons from the surrounding normal tissue. Whether this occurs in gynaecological cancers is unknown. We predict that in gynaecological cancers axons will invade the tumour microenvironment from surrounding tissues under the guidance of neurotrophins. And that axon infiltration will be correlated with clinopathological features.

CHAPTER 2: MATERIALS AND METHODS

2.1. Ovary tissue samples

Tumour microarrays (TMAs) of ovarian cancers and adjacent normal and normal ovarian tissue (T112b and OV20810) were obtained from US Biomax, Inc. (Maryland, USA). OV20810 cohort included 158 serous adenocarcinomas and 35 mucinous adenocarcinomas. In addition, 2 clear cell carcinomas, 2 endometrioid, 1 granular and 4 adenocarcinomas of unknown type were also included. While the small cohort in T112b contained 3 cases of serous papillary adenocarcinoma, 2 each of mucinous adenocarcinoma, clear cell carcinoma and endometrioid adenocarcinoma, 1 granular cell tumour, 2 normal ovary tissue. Both slides include duplicated cores per case. These TMAs proportions are representative of incidence rates of different ovarian tumours reported worldwide (AIHW 2012, Ng, Low et al. 2012). Eighteen samples of normal and normal adjacent to tumour ovarian tissue were also studied. Clinical annotation included tumour size, grade and stage, lymph node invasion and patients' age, registered using TNM grading system as *per* WHO guidelines. Information on treatment and patient outcome was not available. The small core size (1.5mm diameter) is a general limitation of using TMAs. Biomax (USA) quality controls are described as follows. Each single tissue spot on every array slide is individually examined by pathologists certified according to WHO published standardizations of diagnosis, classification and pathological grade. Pathological re-confirmation report is generated and digital image captured. Standard immunohistochemistry tests are also performed to ensure the accuracy and specificity of tissue array products. Each specimen collected

from any clinic was consented to by both hospital and individual. Discrete legal consent form was obtained and the rights to hold research uses for any purpose or further commercialized uses were waived. The study was approved by the Human Research Ethic Committee of the University of Newcastle, Australia.

Table 2.1 – FIGO staging for ovarian cancer (Kurman, Carcangiu et al. 2014, Berek, Crum et al. 2015)

STAGE	DEFINITION
I	Growth limited to the ovaries
II	Growth involves one or both ovaries with pelvic extension
III	Tumour with peritoneal implants outside the pelvis, or positive retroperitoneal or inguinal nodes, or both
IV	Tumour involves one or both ovaries with distant metastasis

2.2. Cervical tissue samples

Tumour microarrays (TMAs) of cervical cancers and adjacent normal and normal cervix tissue (CR6161) were obtained from US Biomax, Inc. (Maryland, USA). This cohort englobed 294 cases of cervical carcinoma with duplicate cores *per* case and 28 adjacent normal and normal tissue in a single tissue core *per* case. The cohort investigated included 257 squamous cell carcinoma cases and 30 adenocarcinomas. Plus, 4 rare cervical adenosquamous carcinomas, 2 clear cell carcinomas and one undifferentiated carcinoma. These proportions are representative of incidence rates of different cervical tumours reported in Australia and worldwide (AIHW 2012). Clinical

annotation included tumour size, grade and stage, lymph node invasion and patients' age. Information on treatment and patient outcome was not available.

Table 2.2 summarizes the staging classification for cancer of the cervix according to the system from The International Federation of Gynaecology and Obstetrics (FIGO).

Table 2.2- FIGO staging for cervical cancer (Kurman, Carcangiu et al. 2014, Bermudez, Bhatla et al. 2015).

STAGE	DEFINITION
0	Pre-invasive disease (carcinoma in situ)
I	Carcinoma strictly confined to the cervix
II	Carcinoma that extends into the parametrium (but not onto the pelvic sidewall) or the upper two thirds of the vagina
III	Carcinoma that has extended onto the pelvic sidewall or involves the lower one third of the vagina. All cases with a hydronephrosis or non-functioning kidney should be included, unless they are known to be due to other causes.
IV	Carcinoma that has extended beyond the true pelvis to distant organs or has clinically involved the mucosa of the bladder, rectum, or both

2.3. Immunohistochemistry

After deparaffinising and hydration of tumour microarrays, antigens were retrieved in a Decloaking Chamber™ at 95°C for 20min or 125°C for 30sec (Table 2.4) in citrate-based low pH buffer (VECTOR Laboratories). Endogenous peroxidases were quenched in 0.3% H_2O_2 (30 min at room temperature) followed by saturation in 2.5% normal

horse serum (ImmPRESS detection kit, VECTOR Laboratories) for 20 min at room temperature. Slides were probed with selected primary antibodies as documented in Table 2.4, or normal rabbit IgG (Alpha Diagnostic, 20009-1-200) at matching isotype concentration or absence of primary antibody, as negative control, in saturating buffer for 1 hour at room temperature. The signal was amplified with horseradish peroxidase-conjugated antibodies anti-rabbit IgG (ImmPRESS detection kit, VECTOR Laboratories) as per manufacturer's recommendations. Immunostaining was visualized with 3, 3-diaminobenzidine chromogen (DAB peroxidase substrate Kit SK-4100, VECTOR Laboratories, USA). Slides were finally counterstained with Gill's formula hematoxylin (VEH-3401, VECTOR Laboratories, USA), dehydrated and cleared in xylene before mounting with Ultramount #4 mounting media (Thermo Scientific).

Table 2.3 documents the TMAs used in this project. Immunolabelling is carried out as *per* our published papers (Pundavela, Demont et al. 2014, Pundavela, Roselli et al. 2015). Antibodies against the various factors / proteins investigated are described in Table 2.4.

Each TMA had embedded controls (adrenal gland (pheochromocytoma) and skin (melanoma)). All antibodies were first tested in *in-house* positive controls (prostate, breast, GIT, DRGs). As negative control, TMA T112b was immunostained, because with duplicated cases (T112b) we could stain half of the slide with the primary antibody of interest and the other half with matching IgG or simply without incubating in primary antisera. Identical approach was done in GIT slides, each containing 2 sections, one immunostained and the other kept as negative control. GIT slides were also used as positive and negative controls while immunostaining all TMAs.

Table 2.3 - Details of human paraffin embedded tissue microarrays (tmas) used in this project.

TMAS Cat. No.1	Tissue microarray description	Cases	Cores
T112B	Ovary cancer tissue array, with normal tissue as control, including TNM*, clinical stage and pathology grade	12	24
OV20810	Ovary cancer survey tissue array (1 of 4), with normal tissue, including TNM* and pathology grade	208	208
CR6161	High-density uterine cervix cancer array, with stage, grade and normal cervical tissue	322	616
HUFPT081	Normal human small intestine	NA	2

* **TNM grading:**

T - Primary tumour
Tx - Primary tumour cannot be assessed
T0 - No evidence of primary tumour
Tis - Carcinoma in situ; intraepithelial or invasion of *lamina propria*
T1 - Tumour invades submucosa
T2 - Tumour invades m*uscularis propria*
T3 - Tumour invades through *muscularis propria* into subserosa or into non-peritonealized pericolic or perirectal tissues.
T4 - Tumour directly invades other organs or structures and/or perforate visceral peritoneum

N - Regional lymph nodes
Nx - Regional lymph nodes cannot be assessed
N0 - No regional lymph node metastasis
N1 - Metastasis in 1 to 3 regional lymph nodes
N2 - Metastasis in 4 or more regional lymph nodes

M - Distant metastasis
Mx - Distant metastasis cannot be assessed
M0 - No distant metastasis
M1 - Distant metastasis

[1] Links
http://www.biomax.us/tissue-arrays/Ovary/T112b
http://www.biomax.us/tissue-arrays/Ovary/OV20810
http://www.biomax.us/tissue-arrays/Uterus/CR6161
http://www.biomax.us/tissue-section-image.php?id=1073

Table 2.4 - Antibodies/markers used to identify axons and/or growth factors in normal and cancerous tissue. Plus, examples of publications in which they were used, and antibody validation done in our laboratories. NGF – Nerve growth factor; GDNF – Glial cell line-derived neurotrophic factor; GFR α1, 2, 3 - GDNF receptor alpha-1, -2 or -3; PGP 9.5 – Protein product gene 9.5; TH – Tyrosine hydroxylase; SP – Substance P; nNOS – (neuronal) Nitric oxide synthase; vAChT – Vesicular acetylcholine transporter; DRG – Dorsal root ganglia, GIT – Gastrointestinal tract.

Neuro marker/ primary antibody	Species raised in	Catalogue number	Final dilution/ concentration used	Antigen retrieval	References	Antibody validation
Growth Factors						
α-NGF	Rabbit	ab52918	1:200	95°C for 20min 90°C for 1min	(Pundavela, Roselli et al. 2015)	IHC WB
α-proNGF	Rabbit	ab9040	0.8mg/ml or 1:200	95°C for 20min 90°C for 1min	(Demont, Corbet et al. 2012, Faulkner, Roselli et al. 2016)	IHC (GIT) WB
GFR – α1, α2, α3	Goat	AF560, AF429, AF2645	5µg/ml	125°C for 30sec 90°C for 10sec	(Forrest, Osborne et al. 2013, Forrest, Payne et al. 2015)	IHC (GIT) / IF (DRG and mouse ovary)
Growth Factors Receptors						
α-TrkA	Rabbit	2508S	1:200	125°C for 30sec 90°C for 10sec	(Lawn, Krishna et al. 2015, Faulkner, Jobling et al. 2017)	IHC (GIT) WB
α-p75^{NTR}	Rabbit	4201S	1:400	125°C for 30sec 90°C for 10sec	(Demont, Corbet et al. 2012)	IHC WB

α-Sortilin	Rabbit	ANT-009	0.8µg/ml or 1:1000	95°C for 20min 90°C for 1min	(Demont, Corbet et al. 2012, Roselli, Pundavela et al. 2015)	IHC
α-TrkB	Rabbit	ANT-019	0.8µg/ml or 1:1000	95°C for 20min 90°C for 1min	(Scott, Zhang et al. 2015)	IHC (GIT) IF
α-NK1	Rabbit	AB5060	1:400	95°C for 20min 90°C for 1min	(Palecek, Paleckova et al. 2003)	IHC (GIT) IF (DRG, GIT, mouse ovary
Nerve fibres or axons						
α-PGP 9.5	Rabbit	ab15503	1:200	95°C for 20min 90°C for 1min	(Pundavela, Roselli et al. 2015)	IHC WB
S100	Rabbit	Z0311	1:1000	95°C for 20min 90°C for 1min	(Rao, Rastelli et al. 2017, Spiric, Eri et al. 2017)	IHC (DRG, mouse mammary gland)
α-TH	Rabbit	AB152	1:500	95°C for 20min 90°C for 1min	(Jobling and Lim 2008, Jimenez-Andrade, Ghilardi et al. 2011)	IHC (prostate) IF (DRG, mouse mammary gland, breast)
Neurotransmitters						
α-vAChT	Goat	AB1578	1:2000	125°C for 30sec 90°C for 10sec	(Brumovsky, Seroogy et al. 2011)	IHC (GIT) IF (DRG, mouse mammary gland)

α-SP	Rat	MAB356	1:50	125°C for 30sec 90°C for 10sec	(Yuan, Gibbins et al. 2011)	IHC (GIT) IF (DRG, mouse mammary gland)
α-nNOS	Rabbit	AB5380	1:500	125°C for 30sec 90°C for 10sec	(Wojtkiewicz, Jana et al. 2014)	IHC (GIT) IF (DRG, mouse mammary gland)

These antibodies have been used for the immunohistochemical identification of the protein's expression pattern in brain, DRG, adrenal gland (pheochromocytoma), skin (melanoma) and GIT slices, and immunoblot experiments; as recorded in table 2.4; reference to representative publications in the same table, as well.

Each marker immunostains the following types of neurons and/or axons:

proNGF & NGF	Sympathetic and sensory neurons
PGP 9.5 & S100	All nerve fibres
TH	Noradrenergic neurons (sympathetic nerves)
SP	Peptidergic neurons (sensory nerves)
vAchT	Cholinergic neurons
nNOS	Nitrigic (sensory) neurons

2.3.1. Antibody optimisation in human gut sections

All antibodies used to identify peripheral nervous system neurons/axons or, growth factors or their receptors, were optimised before use. PGP9.5, TH and NGF antisera that have been used previously in our lab (Pundavela, Demont et al. 2014, Pundavela, Roselli et al. 2015, Faulkner, Roselli et al. 2016). Due to the heterogeneous nature of cancer cells and associated tissue within the tumour microenvironment I optimised and cross-checked antibody labelling in human gut sections #HuFPT081 from US Biomax, Inc. (Maryland, USA) – Figure 2.2, Figure 2.3, Figure 2.4. This allowed us to check the neuronal selectivity of antisera and also ensure we were able to detect the human form of the relevant protein. Some antibodies had previously only been characterised in animal tissue. These included antisera against GDNF alpha receptors 1 and 3 (Schaller, Buttigieg et al. 2017) (Forrest, Osborne et al. 2013) and NK1receptor (Niedermair, Kuhn et al. 2014), commonly used in rodent models of pain. TrkB (ANT-019) was reported in rat models (Schaich, Wellman et al. 2016) and in zebrafish (Gasanov, Rafieva et al. 2015). Likewise, the vAChT antisera has been mainly used in rodents (rat (Ranson, Dowling et al. 2007, Sand, Roth et al. 2014) and guinea pig(Fang, Liu et al. 2008)).

Innervation of the gut is well documented and includes virtually all neurotransmitters found in the peripheral nervous system. In addition, enteric nerves are driven by a range of neurotrophins (Costa, Furness et al. 1982, Brookes 1993, Grundy 1994, Kirkup, Brunsden et al. 2001, Grundy and Schemann 2004, Bornstein 2006, Furness 2006, Arendt-Nielsen, Schipper et al. 2008, Brookes, Zagorodnyuk et al. 2008, Burnstock 2008, Zagorodnyuk, Brookes et al. 2010).

2.4. Image analysis and optical density measurements

Images (8-bit) were captured with an Olympus BX51 microscope (Olympus Australia, Melbourne, Vic) *via* an Olympus DP72 camera controlled by CellSens Standard 1.3 software (Olympus Australia, Melbourne, Vic). The camera exposure settings for each antiserum were chosen to ensure no pixels were saturated and all images were taken at the same exposure to allow semi-quantitative analysis of pixel intensity. The exposure times were determined by the acquisition program which made sure no pixels were saturated and were the same for each TMA. This ensured valid comparisons between different tumour types for each antibody. Once this was set, pixel intensity and area were automated. Plus, presence or absence of labelling was determined by two observers (SO and PJ). Images were analysed using ImageJ 2.0.0 (Schindelin, Arganda-Carreras et al. 2012).

Regions of interest (3.4mm^2) from each TMA were submitted to blind analysis for pixel intensity and area of labelling. Presence of axons (i.e. individual nerve fibres) was scored manually and axon density represented as number of axon profiles per field of view (3.4 mm^2). For NGF-IR quantification, images were processed using the background subtraction algorithm followed by a median filter to eliminate single pixel noise.

In 8-bit digital image analysis, the pixel intensity values for any colour range from 0 to 255 wherein 0 represents the darkest shade and 255 represent the lightest shade. Labelling intensity and area of labelling were registered as a modified h-score where pixel intensity ranging from 0 to 200 was divided into quartiles (3= 0-50, 2= 50-100, 1=100-150, 0 = 150-200). This score was multiplied by labelled area to derive the h-score, as described elsewhere (Rizzardi, Johnson et al. 2012).

2.4.1. Digital quantification with Halo™ software

Immunostaining of TMAs for antisera against NGF and proNGF was analysed through Halo™ image analysis software. TMA slides were digitized at 200x absolute resolution using an Aperio AT2 scanner (Leica Biosystems, Victoria, Australia). Quantitative IHC analyses were performed using the Halo™ image analysis platform (Indica Labs, New Mexico, USA). The pixel intensities of DAB staining were calculated using the Area Quantification algorithm. Pixel intensity values were then used to determine the h-scores for each core (index calculated as the sum of 3 x % of pixels with strong staining + 2 x % of pixels with intermediate staining + 1 x % pixels with weak staining). To compare immunoreactivity levels for each marker across the cohort, the h-scores were used to divide cases into 4 categories (0 = h-score <25, 1 = h-score 25-50, 2 = h-score 50-75, 3 = h-score >75).

2.5. Association between nerve fibres and/or area of staining and clinicopathological parameters

The presence of nerve fibres was compared with clinicopathological parameters (patient age at diagnosis, tumour size, histological subtype, lymph node invasion), and neurochemical marker staining intensity. For statistical analysis, simple unadjusted associations between nerve fibres and other pathological variables were performed using a chi-squared test. In studies of cervical cancer, PGP9.5 core biopsies consisted of 294 tumours and 27 normal samples. For NGF and proNGF core biopsies analysed consisted of 287 tumours and 28 normal cervix samples. Thus, 4 rare cervical

adenosquamous carcinomas, 2 clear cell carcinomas and one undifferentiated carcinoma were not considered for NGF and proNGF analysis. Cervical cancer h-scores data was analysed using t-tests and ordinary one-way ANOVA.

2.6. Cell culture

2.6.1. Cell lines

OVCAR-3 cell line was obtained from Dr Pradeep Tanwar (University of Newcastle, Australia). Cells were maintained in RPMI-1640 medium from Life Technologies (Australia) supplemented with 2mM L-glutamine and 10% foetal calf serum (JRH Biosciences, Lenexa, KS). Immortalized rat dorsal root ganglia neurons 50B11 were obtained from Dr Ahmet Höke (John Hopkins University, Baltimore, MD). 50B11 cells were maintained in Neurobasal, Electro medium with 2% B-27 supplement (A14127-01 Gibco™ by Life Sciences) plus 0.5mM GlutaMAX™-I supplement (Gibco™, Thermo Fisher Scientific Australia). Cultures media were changed about twice a week and cells at ~ 80% confluency were passaged. All cell lines were grown in 75cm^2 tissue culture flasks in a humidified incubator at 37°C with 5% CO_2. The study was approved by the Human Research Ethic Committee of the University of Newcastle, Australia.

2.6.2. Morphology changes of cancer cells grown with neurotransmitters supplementation

Simplified morphological assays were performed with OVCAR-3 cells on 2D for split starved cells. Cell suspensions of 50 000 cells *per* ml of serum-free RPMI-1640

medium with added neurotransmitters NA, ACh and SP (Table 2.5), in a range of concentrations – 0.1, 1, 10, 50 and 100 µM - were added to 12-well plates, and allowed to adhere and grow for 48-72h at 37°C and 5% CO^2 prior to fixation and imaging as described previously.

2.6.3. Preparation of conditioned medium and Transwell invasion / migration assay

Transwell assays are widely used for studying the motility of various types of cancer cells. These assays also allow to screen for compounds that act as chemoattractants or inhibitors of chemotaxis for cells (Justus, Leffler et al. 2014, Katt, Placone et al. 2016) – see Figure 2.1.

In this body of work, cell invasion and migration assays were performed in 24-well Boyden microchambers (Transwell® Permeable Supports) with 8µm pore diameter membranes (#3422, Corning Inc., Cambridge, MA, USA), as previously described (Pundavela, Demont et al. 2014, Pundavela, Roselli et al. 2015, Katt, Placone et al. 2016). For the invasion assay, transwell inserts were first coated with 30µl of rat-tail collagen I matrix (Gibco™, Thermo Fisher Scientific, Victoria, Australia) and incubated overnight at 37°C. The rat-tail collagen matrix was prepared in sterile conditions with collagen-I stock diluted in PBS 1X with 1M NaOH to adjust the pH to an alkaline milieu.

Sub-confluent ovarian cancer cells were starved with 10ml of serum free medium for 24h at 37°C with 5% CO_2. OVCAR-3 cells were then seeded in the top chamber inserts at 70 000 cells *per* ml of serum-free RPMI-1640 medium; 100ul of serum-free medium with cancer cells was loaded in the upper chamber whereas, 600ul of serum-free medium was loaded in to the lower chambers. After 24h, 48h or 62h incubation at

37°C with 5% CO_2, cells having invaded or migrated to the down side of the membrane were fixed and stained with 0.1% (w/v) crystal violet. The whole insert field was counted through an inverted microscope.

Ovarian cancer cells invasion or migration properties were tested with different neurotransmitter formulation diluted in serum-free media loaded into the bottom chamber of the a 24-well plate Transwell plate (#3422 Corning Inc., Cambridge, MA, USA). NA 10µM, ACh 10µM and SP 1µM and 10µM migration and/or invasion effects on OVCAR-3 cells were tested - Table 2.5. This setup will allow to assay the effect of the neurotransmitters in to the cancer cells motility, by chemotaxis, i.e. specifically if the neurotransmitters are chemoattractants or inhibitors of ovarian cancer cell migration.

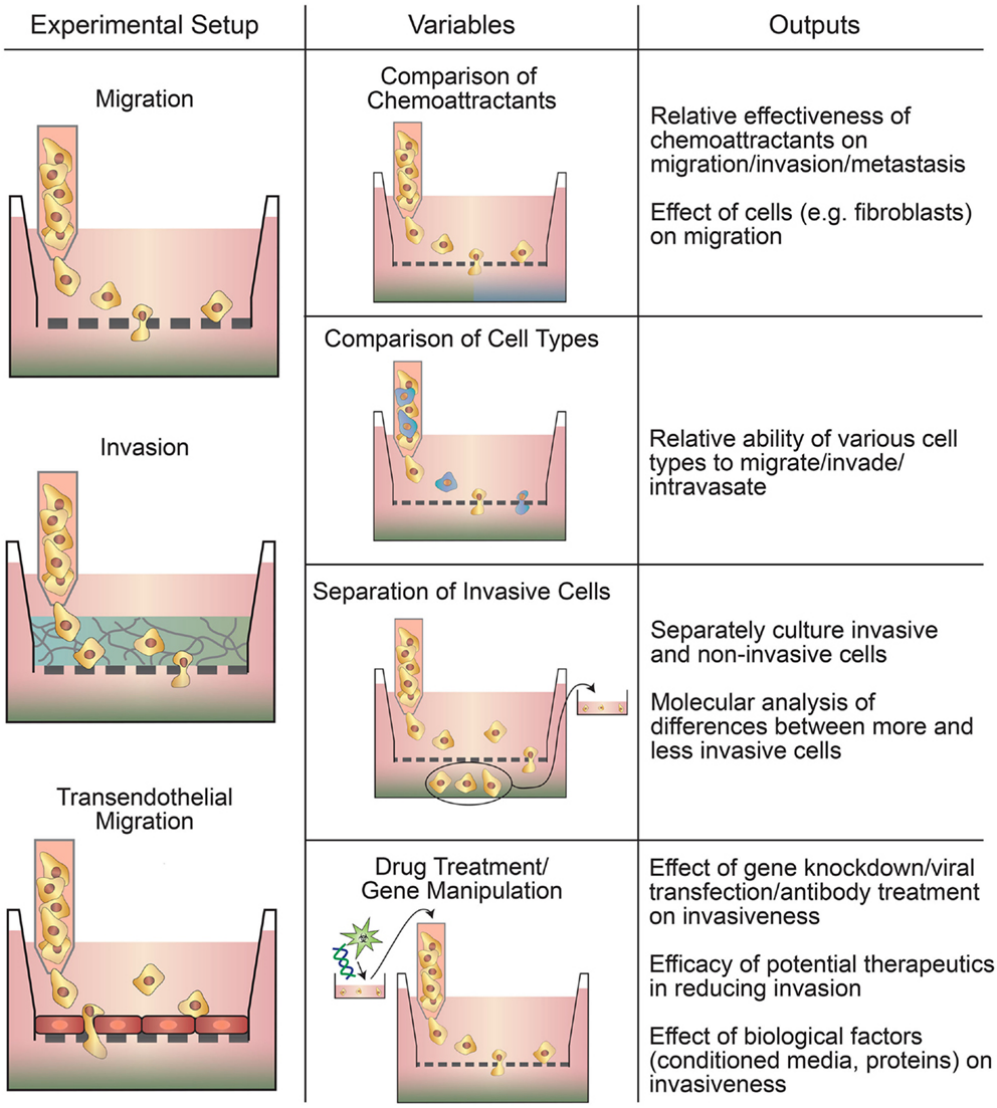


Figure 2.1 – Schematics of the invasion and/or migration assays with Transwell inserts. Migration, invasion and transendothelial migration setups can be used to assess various parameters, including invasiveness and metastatic potential of different type of cells and effects of drugs or gene manipulation on motility. From Katt, Placone et al. (2016).

The multi-well design allows a fast screening process. The cell migration assay measures the number of cells crossing a porous membrane, while the cell invasion assays monitor cell movement through extracellular matrices (e.g. rat-tail collagen I).

Invasive migration is essential for underlying cellular processes such as angiogenesis, embryonic development, immune response, metastasis, and invasion of cancer cells (Justus, Leffler et al. 2014).

2.6.4. Co-culture of immortalized dorsal root ganglia cells 50B11 and ovarian carcinoma cell line OVCAR-3

2.6.4.1. Neurite outgrowth assay

The neurotrophic ability of OVCAR-3 ovarian cancer cell line was tested in co-culture experiments with immortalized dorsal root ganglia cells-like 50B11 and neurite outgrowth and cells morphology was recorded. For OVCAR-3 cells, the experimental setup was identical to the one described previously for the cell migration Transwell™ assays. The 50B11 cells (50 000 cells/ml) were seeded on bottom wells of 12-well Transwell™ plates (#3402 Corning Inc., Cambridge, MA, USA) coated with rat-tail collagen-I (Invitrogen, Australia).

Near confluency, 50B11 cells and OVCAR-3 cells were serum starved in for 24h at 37°C with 5% CO_2. OVCAR-3 cells were grown in Transwell™ inserts (12.0 µm in diameter with 3 µm pores, Corning Inc.). Differentiation of 50B11 cells was allowed for 3 days or until neurites develop, with or without anti-proNGF/NGF mouse monoclonal blocking antibody (alm-006, Alomone, Jerusalem, Israel) and neurite elongation was observed. Controls for neurite elongation were performed with 5µM forskolin and 50µM forskolin (Chen, Mi et al. 2007) and, with proNGF and NGF recombinant protein (Liu, Lamb et al. 2007, Howard, Wyatt et al. 2013) - Table 2.5. 50B11 cells exhibiting neurites of at least twice the size of the cell body were considered as differentiated. Pictures were taken using a Zeiss Axiovert 200 inverted

microscope fitted with an AxioCam HRm digital camera (Zeiss AG). One-way ANOVA statistical test (GraphPad Prism 6) was used.

Table 2.5 - Formulations of neurochemicals used in cell culture assays.

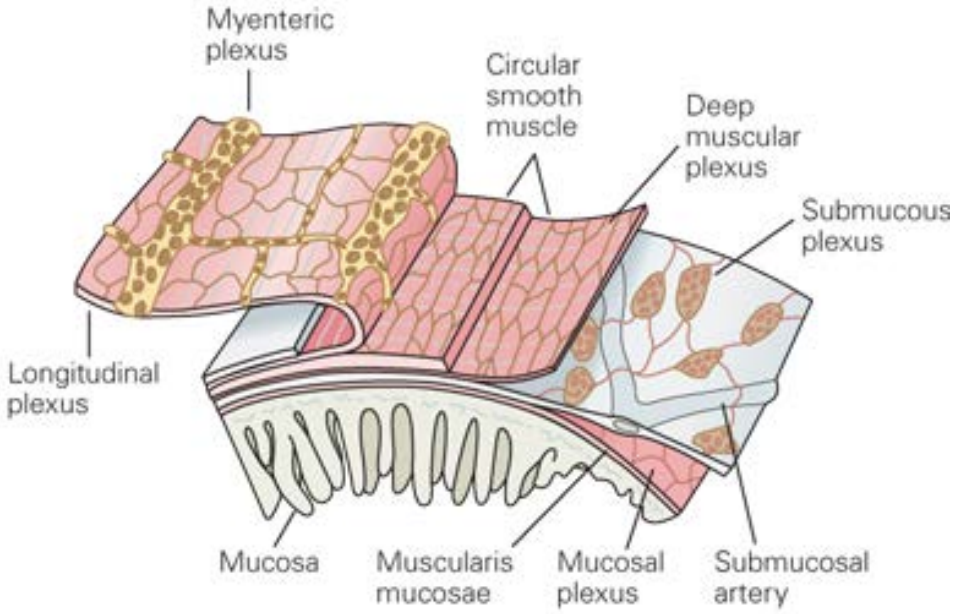
Drug	Catalogue number	Final concentration
DL-Noradrenaline hydrochloride	A7256 Sigma	10µM
Acetylcholine chloride	A-6625 Sigma	10µM
Substance P	2058 AusPep	1µM, 10µM
Forskolin	86-G99 ReagentsDirect	5µM, 50µM
proNGF/NGF blocker antibody	alm-006 Alomone	1µg/ml
proNGF (WT-Human)	N-280 Alomone	50ng/ml
NGF pro-domain (WT-Human)	N-290 Alomone	25ng/ml

2.7. Statistical analysis

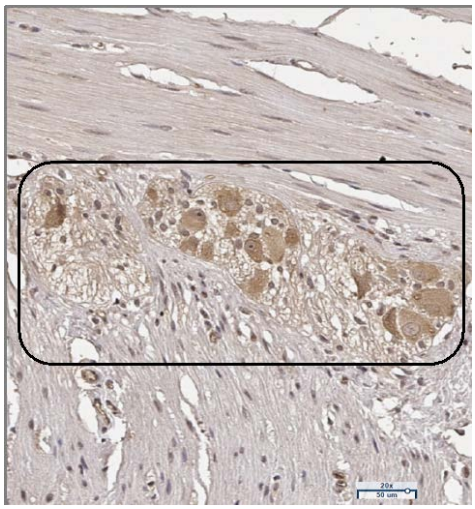
Chi Square analysis followed by Fisher Exact probability test was used for categorical data based on presence or absence of labelling. Axon density was compared using parametric statistics (ANOVA) or Mann-Whitney non-parametric test. For NGF-IR, associations between h-score and clinical presentation were made using a Kruskal-Wallis test followed by a Dunn-Holland-Wolfe test, or a Mann-Whitney test. ANOVA and Tukeys multiple comparison test were performed with cell counting data. Results were considered statistically significant with $p<0.05$. Statistical tests were carried out using GraphPad Prism version 6.0 or 7.0 (GraphPad Software, La Jolla California USA).

Figure 2.2 – Optimisation of neuronal markers in human small intestine. (A) Schematic of the enteric nervous system showing myenteric plexus between the circular and longitudinal muscle layers (in Kandel (2013)). (B) vAChT-IR showing selective labelling of a subpopulation of neurons in the myenteric plexus. (C) SP-IR localised to a subpopulation of neurons in the myenteric plexus. (D) NK1-IR was observed in neurons and within muscle layer and mucosa.

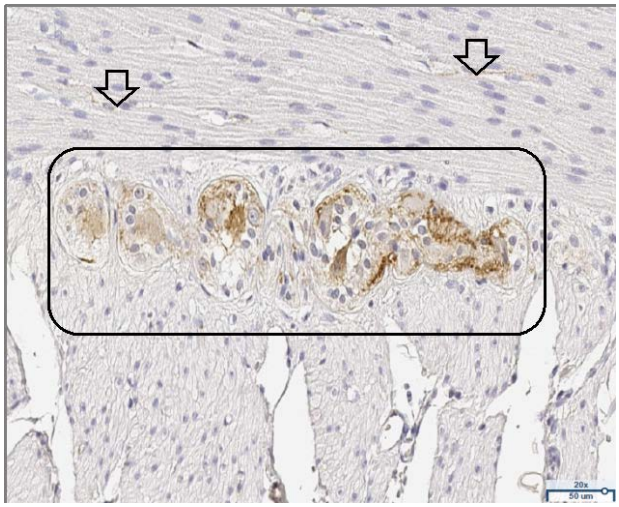
A



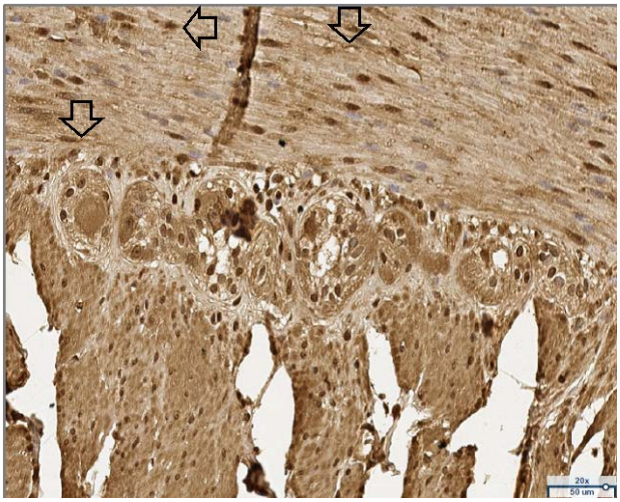
B vAChT



C SP



D NK1



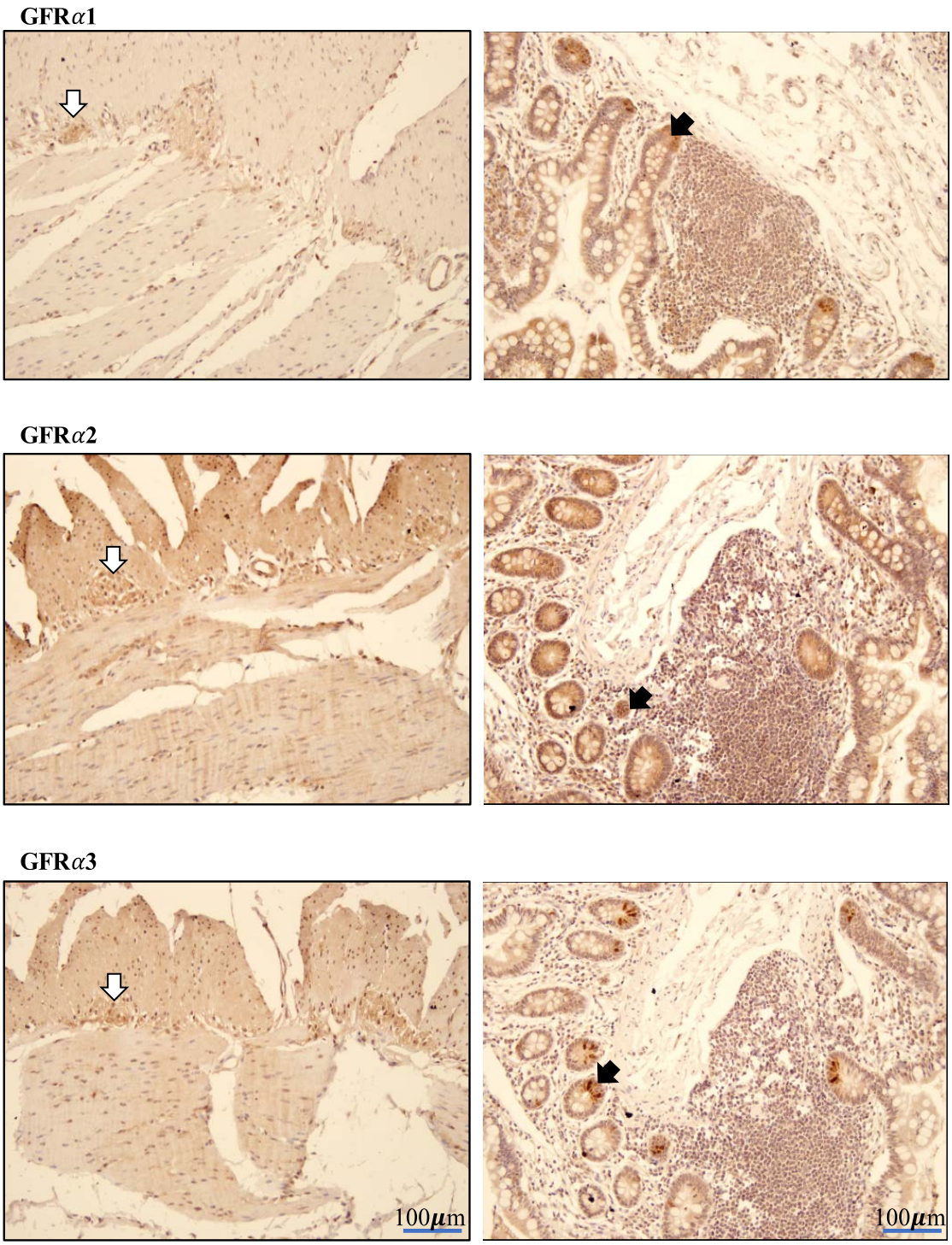


Figure 2.3 – Optimisation of antibodies against GFRs in human small intestine. As expected, immunoreativity to the GFRs was localized to enteric neurons (white arrows) and was also expressed on a subpopulation of epithelial cells (black arrows).

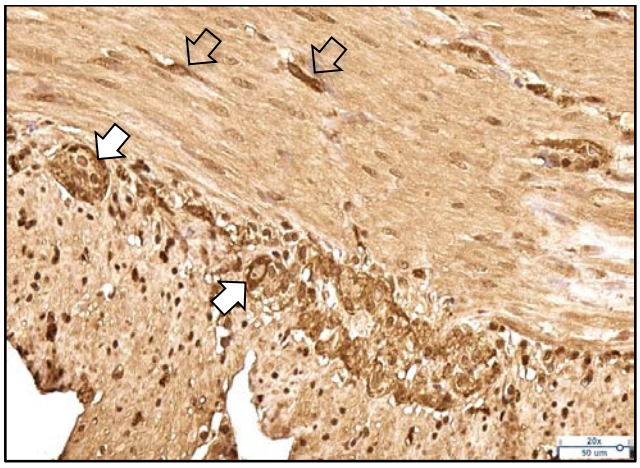


Figure 2.4 – Optimisation of antibodies against nNOS in human small intestine. Immunoreativity to nNOS was localized to enteric neurons (white arrows) and nNOS-IR nerves (arrows).

CHAPTER 3: NERVE INFILTRATION IS A RARE FEATURE IN THE MICROENVIRONMENT OF OVARIAN CANCER

3.1. Background

In the normal ovary, sympathetic nerves are already known to play a major role in the regulation of blood flow and follicle development (Gibson and Roche 1986, Uchida and Kagitani 2015). In addition, the ovary receives sensory axons that presumably send information about the metabolic state of the ovary to the nervous system (Kulkarni, Wakade et al. 1976, Lawrence and Burden 1980, Malishevskaia and Brindak 1980, Erskine and Weaver 1988). Finally, the development of the ovarian sympathetic nerves has been shown to be NGF dependent (Lara, McDonald et al. 1990) and NGF has been reported in some ovarian tumours (Davidson, Reich et al. 2003, Campos, Munoz et al. 2007, Tapia, Gabler et al. 2011). Whether nerves, under the influence of these growth factors, infiltrate ovarian tumours is unknown. Here, we looked for the presence of nerves in the tumour microenvironment of ovarian cancer by immunohistochemistry using the pan-neuronal marker PGP9.5, and the noradrenergic marker TH. I also investigated whether nerve infiltration was correlated with NGF or proNGF expression.

3.2. Materials And Methods

Protocols were as outlined in chapter 2. Briefly:

3.2.1. Ovary tissue samples

Tumour microarrays (TMAs) of ovarian cancers and adjacent normal and normal ovarian tissue (T112b and OV20810) were obtained from US Biomax, Inc. (Maryland, USA). This cohort included 158 serous adenocarcinomas and 35 mucinous adenocarcinomas. In addition, 2 clear cell carcinomas, 2 endometrioid, 1 granular and 4 adenocarcinomas of unknown type were also included.

3.2.2. Immunohistochemistry

Slides were probed with anti-PGP9.5 1:200 (ab15503 from Millipore, USA), anti-NGF 1:200 (ab52918 from Millipore, USA), anti-TH 1:500 (AB152 Millipore, USA), and anti-proNGF 1:200 (ab9040 from Millipore, USA) or normal rabbit IgG (Alpha Diagnostic, 20009-1-200) as a negative control, at 1:200 in saturating buffer for 1 hour at room temperature.

3.2.3. Image analysis and optical density measurements

Images (8-bit) were captured and analysed using ImageJ 2.0.0 (Schindelin, Arganda-Carreras et al. 2012). Regions of interest (3.4mm^2) from each TMA were analysed for pixel intensity and area of labelling. Presence of axons (i.e. individual nerve fibres) was scored manually and axon density represented as number of axon profiles per field of view (3.4 mm^2). For NGF-IR quantification, labelling intensity and area of labelling were registered as a modified h-score where pixel intensity ranging from 0 to 200 was divided into quartiles (3= 0-50, 2= 50-100, 1=100-150, 0 = 150-200). This score was multiplied by labelled area to derive the h-score, as described elsewhere (Rizzardi, Johnson et al. 2012).

3.2.4. Statistical analysis

Chi Square analysis followed by Fisher Exact probability test was used for categorical data based on presence or absence of labelling. Axon density was compared using parametric statistics (ANOVA) or Mann-Whitney non-parametric test. For NGF-IR, associations between h-score and clinical presentation were made using a Kruskal-Wallis test followed by a Dunn-Holland-Wolfe test, or a Mann-Whitney test. Statistical tests were carried out using GraphPad Prism version 6.0 (GraphPad Software, La Jolla California USA).

3.3. Results

3.3.1. The ovarian tumour microenvironment rarely includes nerves

To test if axons of peripheral neurons could infiltrate tumours we labelled tissue microarrays (TMAs) of ovarian cancers versus normal ovarian tissues with the pan-neuronal marker PGP9.5. In normal ovarian tissues, nerves (i.e. bundles of axons) or single axons were found surrounding blood vessels, near follicles and within the stroma (Figure 3.1 A and B). Nerves were observed in 72% of normal ovary sections (Table 3.1, Figure 3.1 A and B).

Tumour sections showed histopathological features consistent with their classification (Cancer 2003, Chen, Ruiz et al. 2003, AJCC 2011, Schueler, Ponnath et al. 2013) (Figure 3.1C and D). Mucinous tumours presented more cystic formations and of larger size compared to serous neoplasms; the cyst's outer surface is smooth with variable solid areas and most cells contain opaque, thick mucoid fluid (Figure 3.1 C).

Serous adenocarcinomas showed tall, columnar epithelial cells, resembling fallopian tube cells, filled with clear serous fluid with many showing papillary projections normally associated with cancer progression and malignancy (Figure 3.1 D). Nerves and axons were found in only 9% of the tumours. Axons were found between cancer cells and surrounding some blood vessels (Figure 3.1 C and D). Protein gene product PGP9.5 is a neuron-specific ubiquitin C-terminal hydrolase. However, it has also been reported in other cell types, including cancer cells (Giambanco, Bianchi et al. 1991, Campbell, Thomas et al. 2003, Otsuki, Yata et al. 2004, Akishima-Fukasawa, Ino et al. 2010). We too observed PGP9.5 expression in non-neuronal cells including ovarian cancer cells (Figure 3.1 D). Prevalence and density (number of axon profiles per field) of nerves were similar in serous versus mucinous tumour types but significantly less than observed in normal ovarian sections (Table 3.1, Figure 3.1 E). Within mucinous or serous tumours, we observed no obvious association between the presence of nerves and tumour size, grade or lymph node invasion.

3.3.2. Sympathetic noradrenergic axons are present in the normal ovary but seldom in ovarian tumours

To investigate the nature of the axons found in the ovarian tumour microenvironment, the TMAs were immunostained with tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine biosynthesis responsible for converting L-tyrosine into L-dopamine (L-DOPA). Nerves and individual axons immunoreactive (IR) for TH were found throughout the stroma of nearly all sections of normal ovary (89% of sections analysed). Axons were observed surrounding blood vessels and adjacent to follicle populations (Figure 3.2 A and B). TH-IR axons were seldom observed in the ovarian tumour microenvironment. Only 7% ovarian tumours contained

TH-IR axons. Of these, 13 were serous adenocarcinomas and 1 was a mucinous adenocarcinoma.

3.3.3. Nerve growth factor (NGF) is expressed in some ovarian tumours but not associated with nerve infiltration.

NGF-IR was observed in 11% of normal ovary sections and in 28% of tumours Table 3.2. NGF-IR was spread throughout the stroma in cancer cells and associated fibroblasts. The proportion of cells labelled (area of labelling) and the intensity of labelling varied considerably between tumours (Figure 3.3 A to D).

When clinicopathological parameters were considered, the proportion of serous and mucinous tumours that expressed NGF-IR were not significantly different between categories. Within serous tumours, NGF-IR did not vary with tumour size Table 3.2 - Association between the presence of nerve growth factor (NGF) and clinicopathological parameters of ovarian tumours.. When tumour grade was investigated, no significant association between grade and NGF-IR was observed either within serous tumours ($\chi 2$ Fisher's exact test, p=0.07), or when tumour type was combined (Table 3.2, p=0.66). No significant association between presence of NGF-IR and lymph node involvement was observed (Table 3.2, p=0.08).

The NGF-IR (calculated as a modified h-score) was close to zero in the normal ovary but varied markedly in cancer with serous ovarian tumours fluctuating between 0 and 30 (Figure 3.4 A). The mean h-score was not significantly different between serous tumours, mucinous tumours and normal tissue (p=0.05) but it was clear that serous tumours showed greater variation in h-score compared with normal and mucinous tumours. Within serous tumours, grade 1 tumours had a greater median h-score compared with grade 2/3 tumours (Figure 3.4 B, Mann-Whitney test p=0.0002).

There was no association between NGF-IR in cancer cells and the presence of nerves in the tumour microenvironment with a similar distribution of axon containing tumours in the NGF – positive vs NGF –negative populations.

3.3.4. ProNGF is expressed in the ovarian tumour microenvironment

ProNGF expression was also analysed in a small cohort of ovarian tumours. TMAs of normal ovary showed proNGF-IR associated particularly with vasculature across ovarian stroma and with primary and secondary follicles (Figure 3.5 C). In parallel, most TMAs of ovarian tumours showed proNGF-IR (80%; Table 3.4). Most of the proNGF detected was centred in ovarian cancer cells and a subset of individual cells in the tumour stroma (Figure 3.5 A and B). Relevant statistical analysis could not be drawn due to the low number of samples; however, it should be noted that proNGF reactivity was not necessarily directly linked to NGF-IR nor to the presence of axons (Table 3.4)

3.4. Discussion

There is increasing evidence for the presence of nerves in solid tumours (Jobling, Pundavela et al. 2015). Most data have been obtained for gastric, prostate and breast cancer (Magnon, Hall et al. 2013, Zhao, Yang et al. 2014, Dobrenis, Gauthier et al. 2015, Pundavela, Roselli et al. 2015). This is the first study to investigate nerves in ovarian cancer. The tumour microarrays studied encompass the most common cancer types and were weighted to serous tumours. Using the pan-neuronal marker PGP9.5, nerves were detected in 9% of the ovarian tumours studied. This is less than half the rate of axon invasion we previously observed in breast tumours (Pundavela, Roselli et

al. 2015). We found no obvious association between the presence of nerves and either histological tumour type or lymph node involvement. This rarity of nerves in ovarian cancers contrasts with our findings in breast tumours (Pundavela, Roselli et al. 2015) and also with reports in prostate cancer where nerve infiltration is highly correlated with cancer aggressiveness (Pundavela, Demont et al. 2014).

While PGP 9.5 is a good marker of all peripheral axons it does not reveal the nature of neurotransmitters released by nerves. Previous studies on normal ovarian innervation suggest that the ovary is supplied by sympathetic and sensory axons. We used the common marker for sympathetic autonomic nerves TH on a subset of TMAs. While TH-positive axons and nerve trunks were regularly observed in the normal ovary, relatively few tumours showed TH-IR. The proportion of tumours with sympathetic nerves was similar to those that expressed the pan-neuronal marker. This suggests that sympathetic axons do infiltrate the ovarian tumour microenvironment, but less commonly than in both prostate and breast tumours (Raju, Haug et al. 2007, Albo, Akay et al. 2011, Magnon, Hall et al. 2013, Zhao, Yang et al. 2014). Given the relatively small contribution of parasympathetic axons to the normal ovarian innervation, we postulate that some of the PGP9.5 immunoreactive axons in ovarian tumours are likely to be sensory, which are abundant in the normal ovary (Kulkarni, Wakade et al. 1976, Burden, Leonard et al. 1983, Papka, Cotton et al. 1985, Ahmed, Dees et al. 1986, Dees, Ahmed et al. 1986, Maggi 1993, Uchida and Kagitani 2014, Wojtkiewicz, Jana et al. 2014).

There was no positive association between nerve infiltration and tumour aggressiveness (as judged by tumour grade and lymph node invasion) in ovarian cancers. In mouse models of prostate cancer, removal of sympathetic nerves, or chemical disruption of catecholamine release markedly slowed tumour growth

(Magnon, Hall et al. 2013), suggesting that catecholamines are promoting tumour growth. Furthermore, a number of cancer cell lines express receptors for neurotransmitters released from autonomic and / or sensory nerves (Al-Wadei, Al-Wadei et al. 2013, Li, Ma et al. 2013, Vilardi, Bravo-Calderon et al. 2013, Kissick, On et al. 2015, Wang, Jin et al. 2015). In gastric cancer, the vagus nerve impacts tumour growth via the stimulation of muscarinic acetylcholine receptors expressed by cancer stem cells (Zhao, Hayakawa et al. 2014). In breast cancer, although the impact of denervation has not been investigated, the presence of nerves in the microenvironment (including TH positive nerves) is associated with lymph node invasion (Pundavela, Roselli et al. 2015). The present study, by showing the rarity of nerves suggests that the concept of nerve infiltration in the microenvironment and its association with tumour aggressiveness does not apply to ovarian cancer.

NGF expression was found expressed in only 2/18 normal ovarian tissue samples with a low h-score. Development of the ovarian sympathetic nerves has been shown to be NGF dependent (Lara, McDonald et al. 1990). NGF also supports the differentiation and survival of the innervating neurons being essential for the gain of the mature ovarian function (Lara, McDonald et al. 1990, Vera, Tapia et al. 2014). However, expression is known to be age and ovarian cycle dependent (Anesetti, Lombide et al. 2001, Dissen, Romero et al. 2001, Romero, Paredes et al. 2002, Ojeda, Paredes et al. 2004, Abir, Fisch et al. 2005, Linher-Melville and Li 2013). We observed NGF-IR in both serous and mucinous tumours but found no obvious correlation with any clinicopathological parameters. If anything, there was a slight reduction of expression in higher grade serous tumours. Campos and colleagues (Campos, Munoz et al. 2007) described NGF expression in ovarian cancer and reported an association with vascular endothelial growth factor (VEGF). However, they did not analyse clinicopathological

parameters. NGF–IR is correlated with the presence of nerves in both breast and prostate tumours (Bradshaw, Pundavela et al. 2014, Pundavela, Roselli et al. 2015). However, in our study, there was no association between positive NGF expression and the presence of nerves in ovarian cancer, further reinforcing the idea that neurogenesis is not a major feature in this malignancy. In fact, the mechanisms that prevent the relatively large numbers of axons in normal ovary from invading tumours may be worth exploring. Moreover, NGF biologically active precursor proNGF presents a more likely valuable piece to understand the pathways that lead to the presence and roles of the autonomic nervous system in the ovarian tumour microenvironment. The role of proNGF in ovarian function and disease remains largely unknown. Only recently NGF and its receptor $P75^{NTR}$ have been pinpointed positively linked to chemotherapy resistance in triple negative breast cancer (Chakravarthy, Mnich et al. 2016), and proNGF proposed as a biomarker in thyroid cancer (Faulkner, Roselli et al. 2016).

In conclusion, although autonomic nerves are abundantly found in the normal ovary, they are not a major feature in the microenvironment of ovarian cancer. Growth-factors such as NGF and proNGF are present in ovarian cancer and their role and mechanisms deserve further exploration. Nonetheless, the concept of a nerve-cancer cell crosstalk that has been described in other human cancers (e.g. prostate), and the therapeutic potential of anti-neurogenic therapies are unlikely to be directly applicable to ovarian cancer.

Table 3.1 - Association between the presence of axons and clinicopathological parameters of ovarian tumours. The pan-neuronal marker PGP9.5 was used to detect axons in the ovarian tumour microenvironment. PGP9.5-IR axons were observed in 19/202 tumour biopsies. But 13/18 histologically normal ovary slide cores also showed axons PGP9.5-IR. Thus, axons were not detected in a significant proportion of cancer tissues and in only a few samples of normal tissue. Of the PGP9.5-IR axons observed in cores of ovarian cancer we found no significative difference in tumour size, grade or lymph node invasion. Over half of the tumours from patients with lymph node involvement expressed PGP9.5. Additionally, the presence of axons was found to be independet of NGF expression.

PARAMETER	NERVE NEGATIVE	NERVE POSITIVE	P-VALUE
All cases (n=220)	188 (85%)	32 (15%)	
Normal (n=18)	5 (28%)	13 (72%)	< 0.0001
Cancer (n=202)	183 (91%)	19 (9%)	
Pathological subtype			
Serous (n=158)	146 (92%)	12 (8%)	0.21
Mucinous (n=35)	30 (86%)	5 (14%)	
Clinical parameters of tumours			
Patient Age			
≤ 50 (n=97)	85 (88%)	12 (12%)	0.16
> 50 (n=105)	98 (93%)	7 (7%)	
Tumour size (T)			
Serous			
T<3 (n=138)	128 (93%)	10 (7%)	0.99
T>3 (n=20)	18 (90%)	2 (10%)	
Mucinous			
T<3 (n=35)	30 (86%)	5 (14%)	NA
T>3 (n=2)	2 (100%)	0	
Tumour grade			
Low (grade 1; n=67)	60 (90%)	7 (10%)	0.80
High (grades 2-3; n=119)	108 (91%)	11 (9%)	
Lymph Node Invasion (N)			
Negative (n=181)	165 (91%)	16 (9%)	0.33
Positive (n=19)	16 (84%)	3 (16%)	
NGF-IR			
Negative (n=145)	133 (92%)	12 (8%)	0.42
Positive (n=57)	50 (88%)	7 (12%)	

Table 3.2 - Association between the presence of nerve growth factor (NGF) and clinicopathological parameters of ovarian tumours. Ovarian tumours express NGF, particularly in the initial tumour stages as per recorded in the tumour size section of the table. But NGF production is not associated with nerve infiltration. Over half of the tumours from patients with lymph node involvement expressed PGP9.5 (Table 3.1). Whilst only a third of these expressed NGF.

PARAMETER	NGF NEGATIVE	NGF POSITIVE	P-VALUE
All cases (n=220)	161 (73%)	59 (27%)	
Normal (n=18)	16 (89%)	2 (11%)	0.12
Cancer (n=202)	145 (72%)	57 (28%)	
Pathological subtype			
Serous (n=158)	110 (70%)	48 (30%)	0.05
Mucinous (n=35)	30 (86%)	5 (14%)	
Clinical parameters of tumours			
Patient Age			
≤ 50 (n=97)	70 (72%)	27 (28%)	0.97
> 50 (n=105)	76 (72%)	29 (28%)	
Tumour size (T)			
Serous			
T<3 (n=138)	94 (68%)	44 (32%)	0.41
T>3 (n=20)	16 (80%)	4 (20%)	
Mucinous			
T<3 (n=35)	30 (86%)	5 (14%)	NA
T>3 (n=0)	0	0	
Tumour grade			
Low (grade 1; n=67)	46 (69%)	21 (31%)	0.62
High (grades 2-3; n=119)	86 (72%)	33 (28%)	
Lymph Node Invasion (N)			
Negative (n=181)	130 (72%)	51 (28%)	0.08
Positive (n=19)	10 (53%)	9 (47%)	
Nerves			
Negative (n=183)	133 (73%)	50 (27%)	0.38
Positive (n=19)	12 (63%)	7 (37%)	

Table 3.3 - Association between the presence of adrenergic (TH-IR) axons and clinicopathological parameters of ovarian tumours.

PARAMETER	TH NEGATIVE	TH POSITIVE	P-VALUE
All cases (n=220)	190 (86%)	30 (14%)	
Normal (n=18)	2 (11%)	16 (89%)	<0.0001
Cancer (n=202)	188 (93%)	14 (7%)	
Pathological subtype			
Serous (n=158)	145 (92%)	13 (8%)	0.47
Mucinous (n=35)	34 (97%)	1 (3%)	
Clinical parameters of tumours			
Patient Age			
≤ 50 (n=97)	91 (94%)	6 (6%)	0.78
> 50 (n=105)	97 (92%)	8 (8%)	
Tumour size (T)			
Serous			
T<3 (n=138)	127 (92%)	11 (8%)	0.39
T>3 (n=20)	17 (85%)	3 (15%)	
Mucinous			
T<3 (n=35)	34 (97%)	1 (3%)	NA
T>3 (n=0)	0	0	
Tumour grade			
Low (grade 1; n=67)	63 (94%)	4 (6%)	0.77
High (grades 2-3; n=119)	109 (92%)	10 (8%)	
Lymph Node Invasion (N)			
Negative (n=19)	9 (47%)	10 (53%)	<0.0001
Positive (n=181)	177 (98%)	4 (2%)	
NGF-IR			
Negative (n=145)	139 (96%)	6 (4%)	0.026
Positive (n=57)	49 (86%)	8 (14%)	
PGP9.5-IR nerves			
Negative (n=183)	171 (93%)	12 (7%)	0.63
Positive (n=19)	17 (89%)	2 (11%)	

Table 3.4 - Association between the presence of proNGF and clinicopathological parameters of ovarian tumours, and correlation to NGF and PGP9.5 immunoreactivity. Whilst we have to be careful about drawing any conclusion due to the small population size, it is interesting that proNGF-IR is detected significatnly in ovarian cancer samples. Pro-NGF expression was associated to NGF expression (78% of cores proNGF-IR positive are also NGF-IR positive), and in a smaller margin to axons PGP9.5-IR positive (60% of ovarian cancer cores with axons expressed proNGF).

PARAMETER	proNGF NEGATIVE	proNGF POSITIVE
All cases (n=12)		
Normal (n=2)	0	2 (100%)
Cancer (n=10)	2 (20%)	8 (80%)
Pathological subtype		
Serous (n=3)	2 (67%)	1 (33%)
Mucinous (n=2)	0	2 (100%)
Clear Cell (n=2)	0	2 (100%)
Endometrioid (n=2)	0	2 (100%)
Granular cell (n=1)	0	1 (100%)
Clinical parameters of tumours		
Patient Age		
≤ 50 (n=9)	1 (10%)	9 (90%)
> 50 (n=1)	1 (100%)	0
Tumour size (T)		
Serous		
T<3 (n=3)	2 (67%)	1 (33%)
T>3 (n=0)	-	-
Mucinous		
T<3 (n=2)	0	2 (100%)
T>3 (n=0)	-	-
Tumour grade		
Low (grade 1; n=4)	1 (25%)	3 (75%)
High (grades 2-3; n=3)	1 (33%)	2 (67%)
Lymph Node Invasion (N)		
Negative (n=9)	2 (22%)	7 78%)
Positive (n=1)	0	1 (100%)
NGF-immunoreactivity		
Negative (n=1)	0	1 (100%)
Positive (n=9)	2 (22%)	7 (78%)
PGP9.5-IR nerves		
Negative (n=5)	0	5 (100%)
Positive (n=5)	2 (40%)	3 (60%)

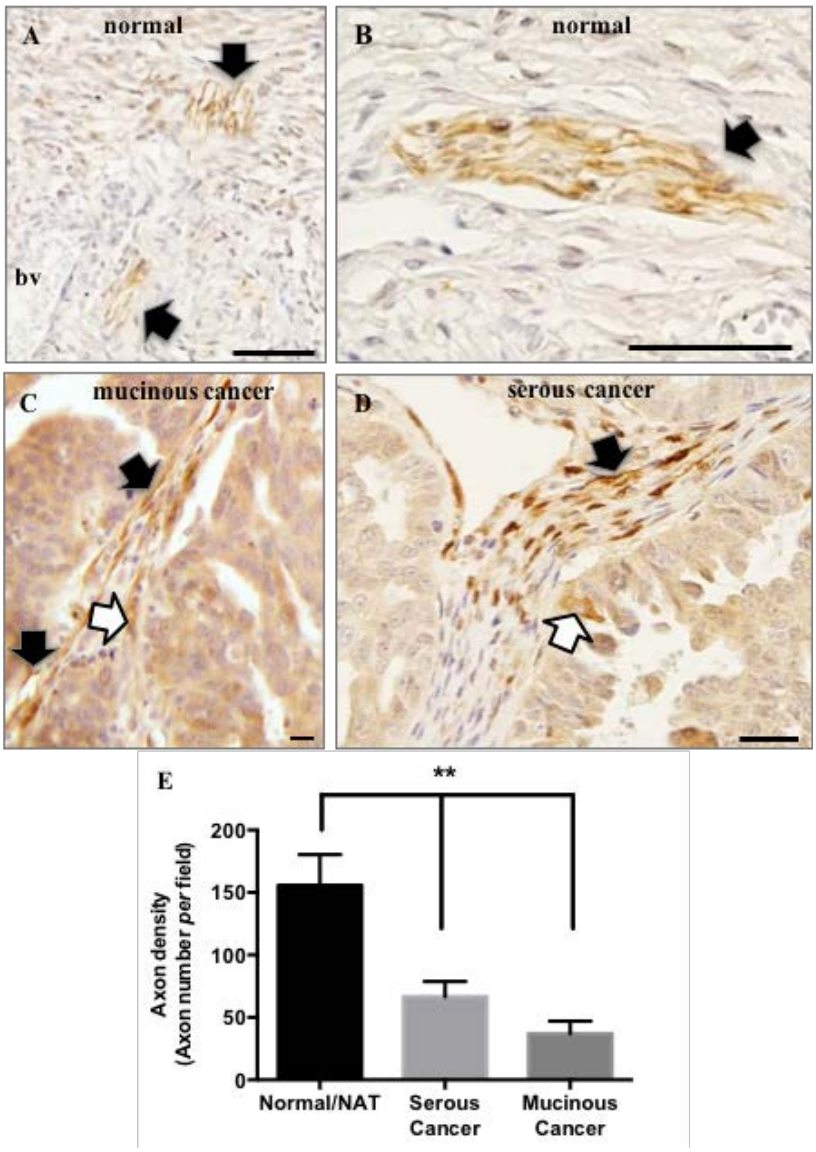


*Figure 3.1 - Detection of nerves in human ovarian tumours using the pan-neuronal marker PGP9.5. (A) Axons (black arrows) were detected in the ovarian stroma and, adjacent to the vasculature (bv) of the normal ovary. (B) Nerve trunks (composed of several axons) were also found in the normal ovary. (C) Mucinous and (D) Serous ovarian tumours showed strong immunoreactivity to PGP9.5 within axons (black arrow), and non-neuronal cells including cancer cells (white arrow). (E) Axon density in the different groups of ovarian tissue microarrays: normal (and normal adjacent to tumour), serous adenocarcinoma and mucinous adenocarcinoma. Data shown as mean ± S.E.M., **p<0.01, one-way analysis of variance with a Dunnett's multiple comparisons test. Field of view 3.4mm2. Scale bar = 50µm.*

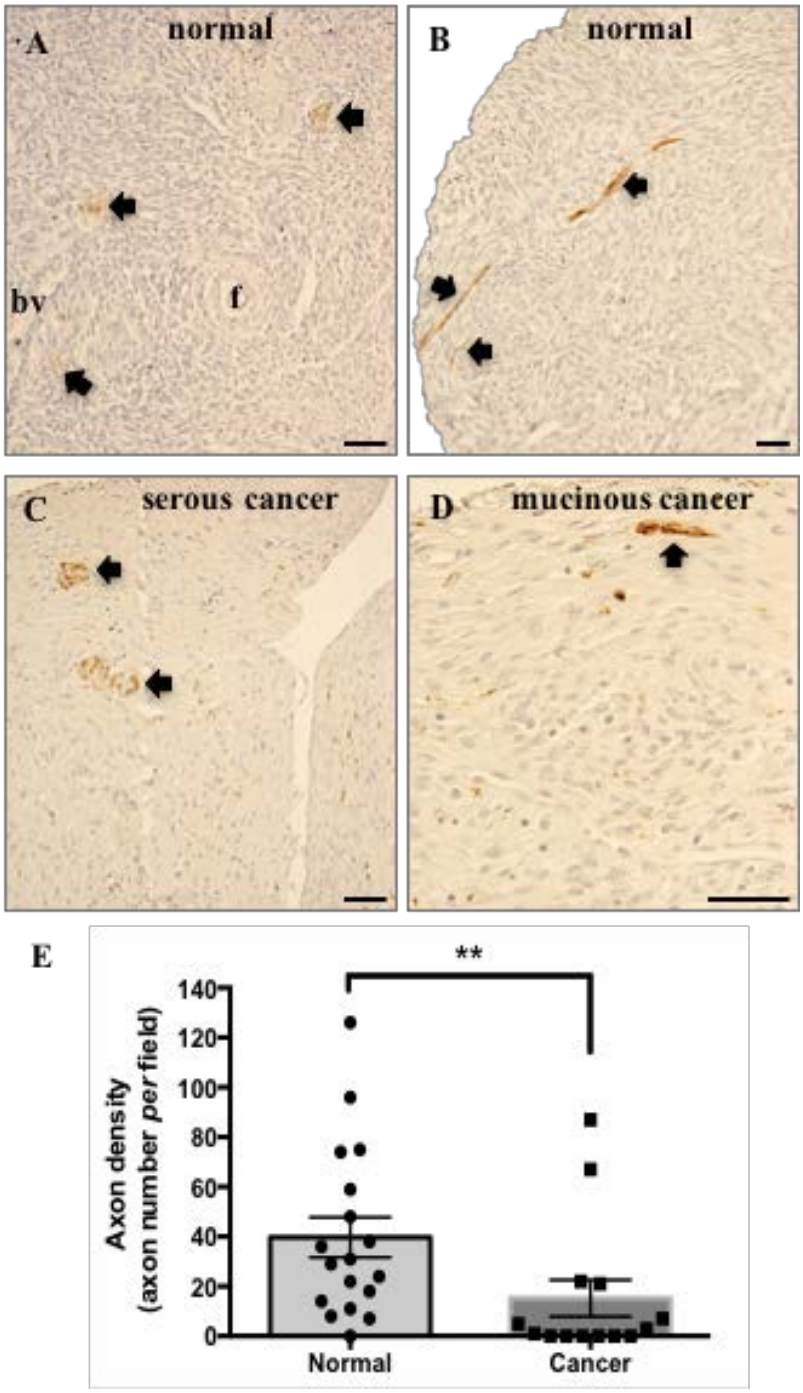


*Figure 3.2 - Detection of sympathetic axons with tyrosine hydroxylase. (A and B) Sympathetic axons (black arrows) coursed throughout the stroma and were often observed near the vasculature. Blood vessel (bv); follicles (f). (C and D) Axons were sparsely distributed in human ovarian tumours. Field of view 3.4mm2. Scale bar = 50µm. (E) Absolute sympathetic axon density in normal and ovarian tumours. Data shown as mean ± S.E.M., **p<0.01, Mann-Whitney test.*

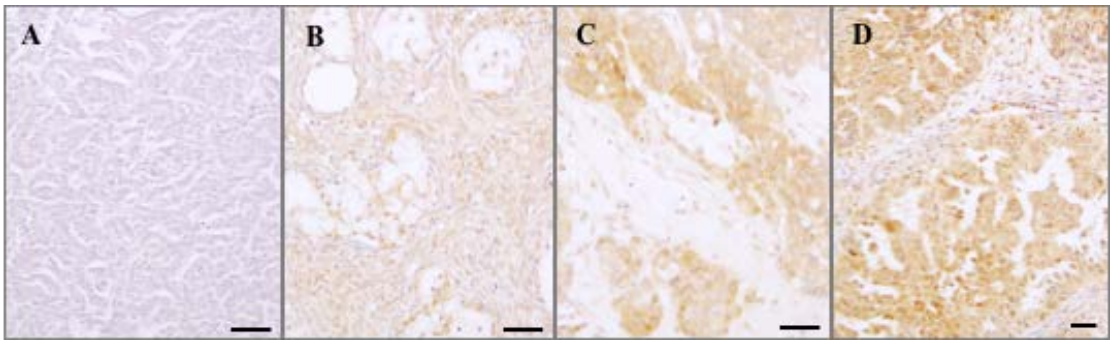


Figure 3.3 - Nerve growth factor (NGF) in the ovarian tumour microenvironment. (A-D) Ovarian tumours showed variable levels of immunoreactivity (IR) from NGF-IR negative (A) to strong NGF-IR (D). Scale bar = 50µm.

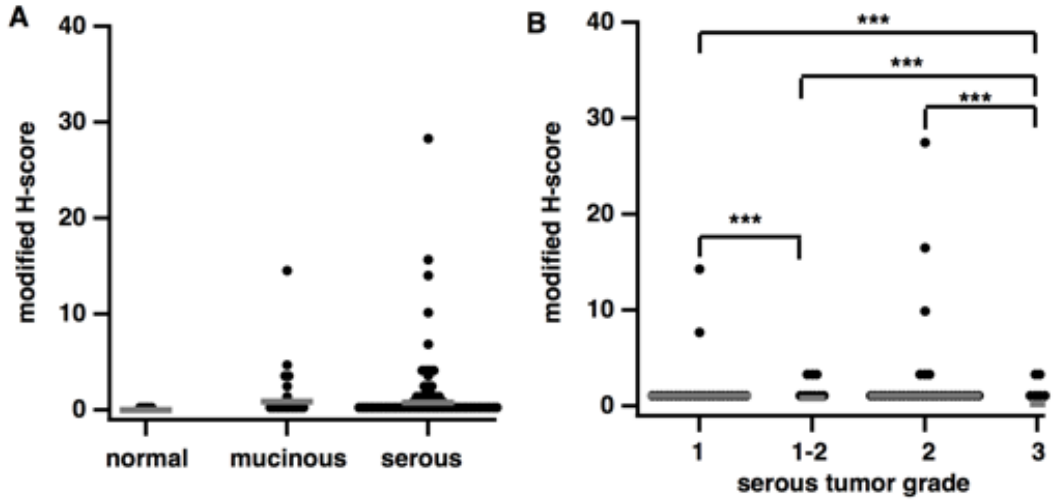
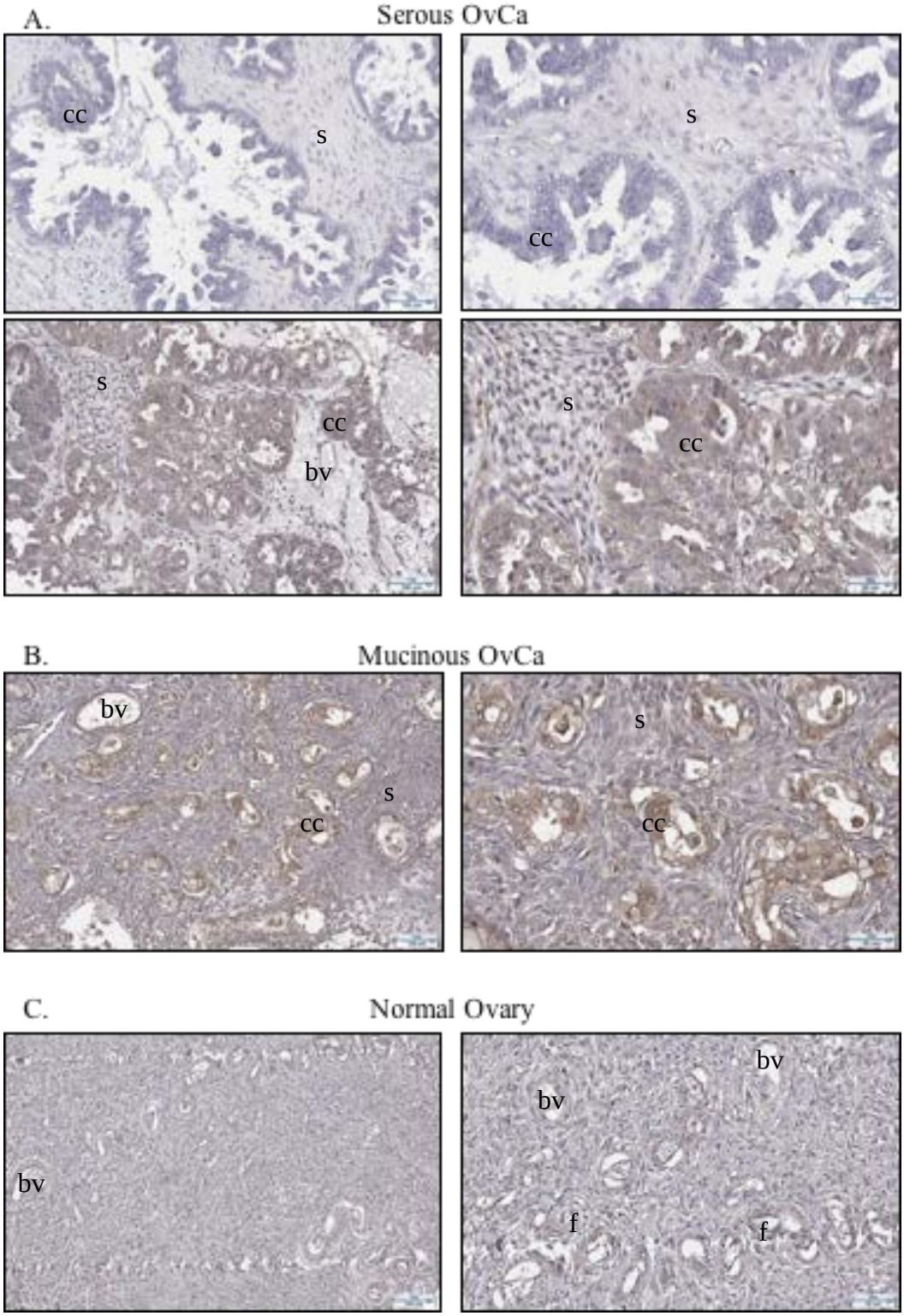


Figure 3.4 - IHC h-scores for NGF-immunoreactivity in a series of ovarian tumours TMAs. (A) h-score had a similar distribution in mucinous and serous tumours. (B) h-score varied with tumour grade (p<0.001) within serous tumours. Low grade serous tumours registered higher median h-score.

Figure 3.5 – Precursor nerve growth factor (proNGF) in the ovarian tumour microenvironment. (A) Serous ovarian tumours (OvCa, ovarian cancer) showed variable levels of immunoreactivity (IR) from proNGF-IR negative (top) to mild proNGF-IR (bottom); (B) Mucinous tumours showed strong proNGF-IR in ovarian cancer cells. (C) Normal ovary showed very low proNGF-IR around initial stage follicles. bv, blood vessel; s, cancer stroma; cc, cancer cells; f, follicle(s).


A.
Serous OvCa
cc
s
s
cc
s
cc
bv
s
cc
B.
Mucinous OvCa
bv
s
cc
s
cc
C.
Normal Ovary
bv
bv
bv
f
f

CHAPTER 4: IDENTIFICATION OF CHOLINERGIC AND PEPTIDERGIC AXONS IN OVARIAN CANCER

4.1. Background

Disruption of neuronal-hormonal processes has been linked to benign conditions of the ovary such as polycystic ovarian syndrome (Dissen, Garcia-Rudaz et al. 2009, Saller, Merz-Lange et al. 2012). However, the innervation of ovarian cancer remains overlooked.

I previously observed nerve infiltration in ovarian tumours using the general marker PGP 9.5 (CHAPTER 3:). I then looked for such adrenergic nerves using TH antiserum to discover that these were not a major component of the ovarian tumour microenvironment. Therefore, I hypothesized that other populations of peripheral nerves may be infiltrating the tumour microenvironment. As outlined in chapter 1 potential candidates may be cholinergic nerves or one of the unmyelinated sensory nerve subtypes. My aim in this chapter was to look for the presence of axons immunoreactive for vACHT or SP in ovarian tumours. In addition, I looked for immunoreactivity to the NK1 receptor in a small subset of tumours.

4.2. Materials And Methods

Protocols were as outlined in chapter 2.

4.2.1. Ovary tissue samples

Tumour microarrays (TMAs) of ovarian cancers and adjacent normal and normal ovarian tissue (T112b and OV20810) were obtained from US Biomax, Inc. (Maryland, USA). This cohort included 158 serous adenocarcinomas and 35 mucinous adenocarcinomas. In addition, 2 clear cell carcinomas, 2 endometrioid, 1 granular and 4 adenocarcinomas of unknown type were also included. Eighteen samples of normal and normal adjacent to tumour ovarian tissue were also studied.

4.2.2. Immunohistochemistry

After optimising two VACHT and one CHAT antisera in our positive control tissue (ileum) we chose anti-vAChT 1:2000 (AB1578 Millipore, USA) as our cholinergic marker. For putative peptidergic signalling, anti-SP 1:50 (MAB356 Millipore, USA) and anti-NK1 1:400 (AB5060 Millipore, USA) were used. Normal goat or rat IgG, respectively (Alpha Diagnostic) were used as negative controls at 1:200.

4.2.3. Statistical analysis

Chi-Square analysis was used for categorical data based on presence or absence of labelling. Statistical tests were carried out using GraphPad Prism version 6.0 (GraphPad Software, La Jolla California USA).

4.3. Results

4.3.1. The ovarian tumour microenvironment includes cholinergic signalling

To test if cholinergic axons could infiltrate ovarian tumours tissue microarrays (TMAs) of ovarian cancers and normal ovarian tissue were labelled with antisera against vAChT. In normal ovarian tissues, vAChT-IR nerves (i.e. bundles of axons) or single axons were found surrounding follicles and blood vessels (Figure 4.1 C). vAChT-IR including axons was observed in 72% of normal ovary sections (Table 4.1 C). Tumour sections showed histopathological features consistent with their classification (Cancer 2003, Chen, Ruiz et al. 2003, AJCC 2011, Schueler, Ponnath et al. 2013) (Figure 4.1). Nerves and axons were found in 98/202 (49%) tumours. But 78% of tumours showed vAChT-IR also in other cell types such as in cancer cell membranes and neuroendocrine cells (Table 4.1; Figure 4.1A, B and D). Axons were found between cancer cells and occasionally in the cancer stroma and blood vessels; vAChT-IR was also observed in cancer cells membranes (Figure 4.1 A and B). Prevalence of nerves were comparable in serous *versus* mucinous tumour types (Table 4.1, Figure 4.1 D). Within mucinous or serous tumours, we observed no obvious statistical association between the presence of nerves and tumour size, grade or lymph node invasion.

4.3.2. Substance P peptidergic axons are not a major feature in normal and ovarian cancer

To further investigate the nature of the axons found in the ovarian tumour microenvironment, TMAs were immunostained with SP, a neuropeptide of the

tachykinin family that acts as a neurotransmitter and neuromodulator after release from the terminals of specific sensory nerves.

Most SP-IR was associated to small neuroendocrine-like cells and small axons often observed in clusters (Figure 4.2). SP-IR was found throughout the stroma of a small number of sections of normal ovary (39%; Table 4.2). Neuroendocrine cells were found in 17% of the ovarian tumours observed (Table 4.2, Figure 4.2 A and B) mostly near blood vessels and in the tumour stroma. Similarly, small SP-IR cells were observed in the tumour stroma. SP immunoreactivity was expressed a greater number of serous tumours compared with mucinous carcinomas (p=0.0158; Table 4.2).

Although a number of tumours did express SP-IR, the relative density of axons or putative neuroendocrine cells was very low ranging from 1-5 mostly near blood vessels. SP-IR was not a significant feature in the ovarian tissues observed - normal or carcinoma - and was not associated with axonal immunostaining (Figure 4.2 D). Normal ovary showed more SP-IR TMAs than ovarian tumours (p=0.0257; Table 4.2). SP-IR axons were seldom observed in the normal ovary (Figure 4.2 D) while a number of SP-IR axons were observed in ovarian tumours (Figure 4.2 B).

4.3.3. Neurokinin 1 (NK1) is highly expressed in some ovarian cancer cells

The presence of SP endogenous receptor, Tachykinin NK1 receptor was also investigated in a small number of samples (Biomax, Inc. T112b slide). In contrast to SP-IR, NK1 receptors were often detected in both normal and cancerous ovary (Figure 4.3). Nk1-IR was high around the vasculature (Figure 4.3 C) and in some cancer cells (Figure 4.3 B). NK1-IR was detected also in axons running through serous ovarian cancer cells (Figure 4.3 B). The intensity of staining varied across tumours (Figure 4.3).

However, in the normal ovary NK1 was exhibited running through the ovarian stroma and highly associated with blood vessels and some early stage follicles (Figure 4.3 C). No relationship was established between NK1-IR and any clinicopathological parameters except for patients' age, where younger patients may have more tendency to express this transmembrane receptor (p=0.0350; Table 4.3).

No relationship was found between SP-IR and NK1-IR (p=0.7469; Table 4.3).

4.3.4. nNOS-IR in ovarian cancer

nNOS-IR was observed in cancer and normal ovary. In a small TMA consisting of 2 normal and 10 ovarian tumours, nNOS-IR was observed in all cases. Immunoreactivity was not restricted to nerves but was widely expressed in cancer cells and throughout the stroma from all cancer subtypes, although the level of expression varied between cancers (Figure 4.4 A and B). In both the normal ovary cases, nNOS-IR was also observed in non-neuronal cells, and not restricted to nerves (Figure 4.4 C and D).

4.4. Discussion

The importance of the nervous system in organ function and pathology has been increasingly reported (Albo, Akay et al. 2011, Mancino, Ametller et al. 2011, Demir, Friess et al. 2012, Jobling, Pundavela et al. 2015). Cholinergic signalling has been shown to play a major role in prostate cancer and implicated in gastric cancer (Magnon, Hall et al. 2013, Zhao, Hayakawa et al. 2014). Furthermore, SP signalling has been linked to cancer proliferation *in vitro* (Garcia-Recio, Fuster et al. 2013, Li, Ma et al. 2013). In this study, we aimed to investigate the cholinergic or peptidergic (SP)

innervation of ovarian cancer. The tumour microarrays studied encompass the most common ovarian cancer types and are proportionally harmonised to the statistics of diagnostic worldwide.

vAChT –IR is generally considered a good tool for the study of cholinergic neurons in the central and peripheral nervous systems (Arvidsson, Riedl et al. 1997). However, cholinergic signalling has been observed in other systems, particularly immune tissue where ACh is known to regulate macrophage and lymphocyte function (Theodorou, Fioramonti et al. 1996, Hu and McLachlan 2002, Hu and McLachlan 2003, Lang, Drell et al. 2003, Nance and Sanders 2007, Rosas-Ballina, Ochani et al. 2008). Moreover, a cholinergic system has been reported in other non-neuronal tissues such as epithelia, and in spermatogenesis (Schirmer, Eckhardt et al. 2011).

In an ovarian context, recent data has also shown that ACh potentiates the growth and differentiation of ovarian follicles contributing to ovulation and fertility (Urra, Blohberger et al. 2016). Cholinergic nerves have also been linked to ovarian steroidogenesis and their density may be altered in models of polycystic ovary disease (Kozlowska, Wojtkiewicz et al. 2013, Kozlowska, Majewski et al. 2014, Wojtkiewicz, Jana et al. 2014, Delsouc, Morales et al. 2016). In normal ovary, I found vAChT-IR was mostly associated with primordial and primary follicles but also within blood vessels. This is consistent with previous reports of ACh production by granulosa cells of growing follicles and luteal cells (Sporrong, Kannisto et al. 1985, Kozlowska, Wojtkiewicz et al. 2013, Kozlowska, Majewski et al. 2014).

While some ovarian tumours did not show any immunoreactivity to vAChT antisera (22% of the tumours analysed), most ovarian carcinomas expressed vAChT from moderate to intense, inclusively in cancer and in immune cells. The expression of

VACHT-IR in a subset of cells in a subset of core biopsies on the same slide suggests that our antibody is selective. However, without the resolution of an electron microscope we cannot determine if vAChT-IR is associated with vesicular release. Therefore, the functional implications of the non-neuronal expression of vAChT are unknown. Other cholinergic markers may need to be used to probe non- neuronal signalling. Nevertheless, we have opened up the possibility that cholinergic mechanisms from both neuronal and non-neuronal sources are at play in ovarian cancer. Such has also been suggested in other solid tumours as in prostate cancer (Chapple, Crowe et al. 1991, Ventura, Pennefather et al. 2002, Witte, Chapple et al. 2008, Magnon, Hall et al. 2013)

I also considered sensory peptidergic innervation in the ovary in order to assess innervation in the ovarian tumour microenvironment. Although a number of other unmyelinated sensory neuron populations are likely to be present SP containing neurons are a feature of most viscera and have been described in other mammals within the ovary (Papka, Cotton et al. 1985, Klein and Burden 1988, Schultea, Dees et al. 1992). Furthermore, a link between SP-NK1 pathway and cancer migration/progression has been suggested (Seckl, Higgins et al. 1997, Munoz and Covenas 2013).

I observed SP-IR cells and axons in the ovary, both in normal and cancer but at a very low density suggesting that their role is rather limited in the aetiology and progression of ovarian cancer. SP-IR is higher in serous carcinomas than in mucinous, but more frequently observed in the normal ovary than in ovarian tumours. This is the first description of SP neurotransmitter in ovarian cancer. Data has been described in polycystic ovaries, where unlike their normal ovarian histology controls, cystic ovaries samples showed perivascular SP-axons in medullar blood vessels (Kozlowska,

Wojtkiewicz et al. 2011, Wojtkiewicz, Jana et al. 2014). We observed proportionally more SP-IR in the normal ovary than in cancer, however also mostly in the ovarian and cancer stromas, and nearby blood vessels. Limiting the significance of this observation lies the knowledge that the histologically normal samples were taken from ovaries with tumours thus from areas adjacent to tumour but of normal histology. Despite the relatively low density of SP-IR in tumour biopsies, we observed a relatively high expression of the tachykinin NK_1 receptor. Although the NK1 receptor has the highest affinity for SP, other tachykinins may activate this receptor (Iversen 1994, Jobling, Messenger et al. 2001).

A search for other endogenous ligands may prove fruitful. Therefore, we further look for the main SP endogenous receptor, NK_1 receptor that together with SP form a key first response to most noxious or stress stimuli. SP-NK1 signalling pathways are often linked to proliferative and inflammatory signalling pathways in different tissues and conditions, from obstructive sleep apnoea to chagasic megacolon (da Silveira, Freitas et al. 2008, Gozal, Kim et al. 2014). SP, and its receptor NK1, roles in ovulation, spermatogenesis and steroidogenesis including the hypothalamic-pituitary-gonadal axis have been discussed (Lasaga and Debeljuk 2011).

NK1-IR was observed in a small sample of human ovarian tumours and found it to be expressed in the majority of cases, particularly in cancer cells although not all tumours had the same level of expression. Interestingly, normal ovarian tissues showed NK1-IR nerve fibres, namely within blood vessels and primordial and primary follicles. NK1-IR has previously been described in autonomic (Jobling, Messenger et al. 2001) and sensory neurons (von Banchet and Schaible 1999). It should be noted that although SP is the preferred ligand for NK1 receptors they can be also activated by neurokinin

A and neurokinin B (Alexander, Davenport et al. 2015).

nNOS-IR is not restricted to nerves in either cancer or normal ovary. Similar to previously published reports nNOS-IR were observed in normal ovarian follicles (Wojtkiewicz, Jana et al. 2014). However, we also observed nNOS in non-neuronal cells in both cases of normal ovary. In the cancer samples, nNOS was even more widely expressed in non-neuronal cells. Whether this represents staining through neuronal NOS or a protein with a epitope recognize by the nNOS antibody is unclear. Different antisera against NO should be useful in future investigations. Although our nNOS antibody was selective for neurons in the small intestine (chapter 2).

Table 4.1 - Association between the presence of vAChT and clinicopathological parameters of ovarian tumours. 78% of ovarian tumours showed some sort of immunoreactivity to vAChT antisera (ie. cancer cells, neuroendocrine or immune cells or axons). 98 ovarian tumour cores out of 158 cores expressing vAChT showed cholinergic innervation. However, a total of 8 normal ovary samples showed vAChT stained axons, out of 13 normal samples recorded expressing vAChT. Adittionally, younger patients are more likely to express vAChT in the ovarian cancer tissue (p=0.0365). vAchT expression also showed some tendency to be higher in mucinous tumours (p=0.2362) and/or smaller size serous tumours (ie non-metastatic tumours, T<3 of TNM grading (p=0.1693)). The later, matches with a higher likehood of vAChT expressing tumours not be lymph-node invaded (p=0.2450).

PARAMETER	vAChT NEGATIVE	vAChT POSITIVE	P-VALUE
All cases (n=220)			
Normal (n=18)	5 (28%)	13 (72%)	0.5580
Cancer (n=202)	44 (22%)	158 (78%)	
Pathological subtype			
Serous (n=158)	37 (23%)	121 (77%)	0.2362
Mucinous (n=35)	5 (14%)	30 (86%)	
Clinical parameters of tumours			
Patient Age			
≤ 50 (n=97)	15 (15%)	82 (85%)	**0.0365**
> 50 (n=105)	29 (28%)	76 (72%)	
Tumour size (T)			
Serous			
T<3 (n=138)	35 (25%)	103 (75%)	0.1693
T>3 (n=20)	8 (40%)	12 (60%)	
Mucinous			
T<3 (n=35)	7 (20%)	28 (80%)	-
T>3 (n=0)	-	-	
Tumour grade			
Low (grade 1; n=67)	17 (25%)	50 (75%)	0.8790
High (grades 2-3; n=119)	29 (24%)	90 (76%)	
Lymph Node Invasion (N)			
Negative (n=183)	45 (25%)	138 (75%)	0.2450
Positive (n=19)	7 (37%)	12 (63%)	
vAChT-IR nerves			
Nerves Negative	42	50	
Nerves Positive	-	98	
vAChT-IR nerves in normal ovary			
Nerves Negative	5	13	
Nerves Positive	-	8	

Table 4.2 - Association between the presence of substance P (SP) and clinicopathological parameters of ovarian tumours. The data showed that tissues of histologically normal ovary are more likely to express SP than cancer (p=0.0257). Furthermore, more serous ovarian tumours showed SP-IR than mucinous tumours (p=0.0158). No link was found with other clinical parameters of the tumours.

PARAMETER	SP NEGATIVE	SP POSITIVE	P-VALUE
All cases (n=220)			
Normal (n=18)	11 (61%)	7 (39%)	**0.0257**
Cancer (n=202)	167 (83%)	35 (17%)	
Pathological subtype			
Serous (n=158)	127 (80%)	31 (20%)	**0.0158**
Mucinous (n=35)	34 (97%)	1 (3%)	
Clinical parameters of tumours			
Patient Age			
≤ 50 (n=97)	82 (85%)	15 (15%)	0.5014
> 50 (n=105)	85 (81%)	20 (19%)	
Tumour size (T)			
Serous			
T<3 (n=138)	112 (81%)	26 (19%)	0.5168
T>3 (n=20)	15 (75%)	5 (25%)	
Mucinous			
T<3 (n=35)	34 (97%)	1 (3%)	-
T>3 (n=0)	-	-	
Tumour grade			
Low (grade 1; n=67)	58 (87%)	9 (13%)	0.3746
High (grades 2-3; n=119)	97% (82%)	22 (18%)	
Lymph Node Invasion (N)			
Negative (n=183)	153 (84%)	30 (16%)	0.2767
Positive (n=19)	14 (74%)	5 (26%)	

Table 4.3 - Association between the presence of NK1 receptors and clinicopathological parameters of ovarian tumours. No significant differences were found between NK1 expression and patients' clinical data obtained. We draw no conclusions from p=0.0350 between patients age and NK1-IR due to the small population analysed (n=10 ovarian tumours). Although most SP-IR positive tumours also expressed NK1, there was not a direct correlation between them.

PARAMETER	NK1 NEGATIVE	NK1 POSITIVE	P-VALUE
All cases (n=12)			
Normal (n=2)	0	2 (100%)	0.4884
Cancer (n=10)	2 (20%)	8 (80%)	
Pathological subtype			
Serous (n=3)	1 (33%)	2 (67%)	0.3613
Mucinous (n=2)	0	2 (100%)	
Clinical parameters of tumours			
Patient Age			
≤ 50 (n=9)	1 (11%)	8 (89%)	**0.0350**
> 50 (n=1)	1 (100%)	0	
Tumour size (T)			
Serous			
T<3 (n=3)	1 (33%)	2 (67%)	-
T>3 (n=0)	-	-	
Mucinous			
T<3 (n=2)	0	2 (100%)	-
T>3 (n=0)	-	-	
Tumour grade			
Low (grade 1; n=4)	0	4 (100%)	0.2123
High (grades 2-3; n=3)	1 (33%)	2 (67%)	
Lymph Node Invasion (N)			
Negative (n=9)	2 (22%)	7 (78%)	0.5982
Positive (n=1)	0	1 (100%)	
SP immunoreactivity			
Negative (n=6)	1 (17%)	5 (83%)	0.7469
Positive (n=4)	1 (25%)	3 (75%)	

Figure 4.1 – vAChT-IR in ovarian cancer and normal ovary. (A) Serous tumours showing vAChT-IR in both cancer cells and stroma. A.2) shows a higher magnification of cancer cells showing presumpted axon bundle (red circle). (B) Mucinous tumours showing vAChT-IR in cancer cells but not stroma. B.2) shows higher magnification of vAChT-IR in cancer cells and presumpted neuroendocrine cells (red circle). (C) vAChT-IR in normal ovary restricted to nerves adjacent to follicles. C.2) shows a higher magnification of vAChT-IR axons adjacent to primary follicles. (D) Quantification of the number of cases showing either no vAChT-IR (vAChT -), vAChT-IR expressed in cancer cells or nerves (vAChT +), or patients with vAChT-IR nerves. Scale bar = 50µm

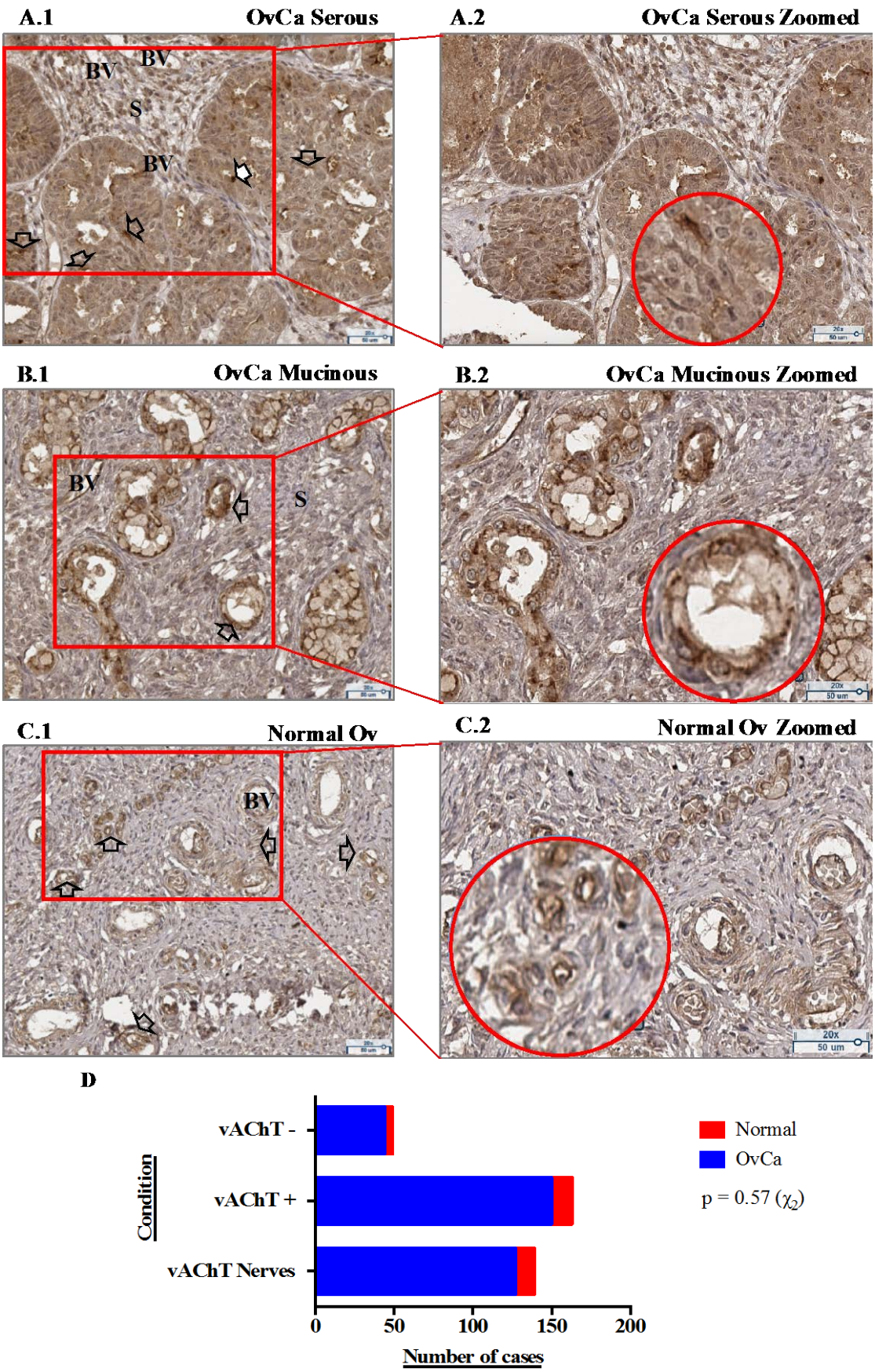
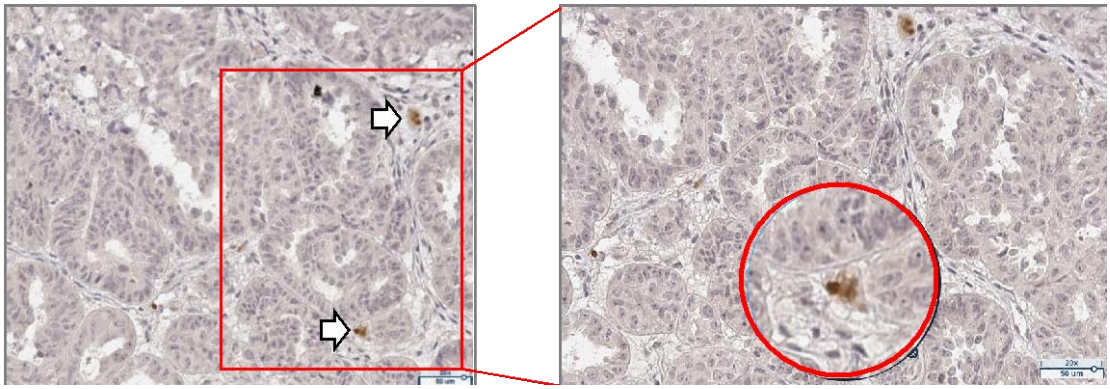
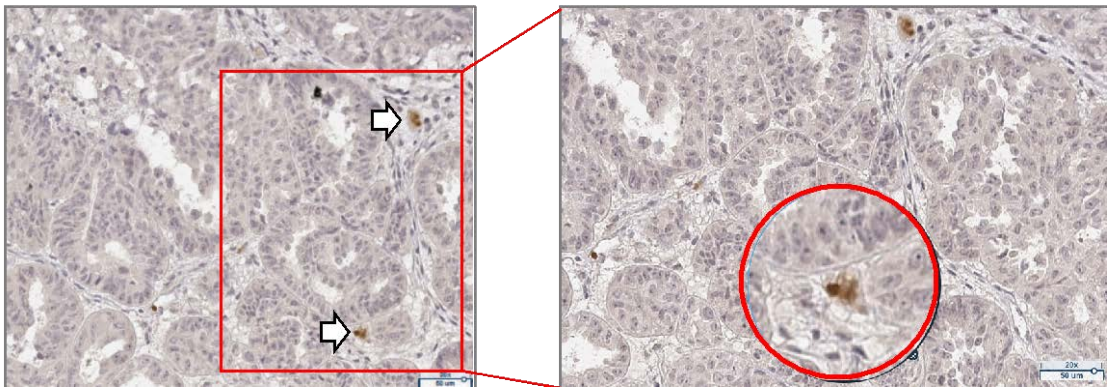

A.1
OvCa Serous
BV
BV
S
BV
A.2
OvCa Serous Zoomed
B.1
OvCa Mucinous
BV
S
B.2
OvCa Mucinous Zoomed
C.1
Normal Ov
BV
C.2
Normal Ov Zoomed
D
Condition
vAChT -
vAChT +
vAChT Nerves
0
50
100
150
200
Number of cases
Normal
OvCa
p = 0.57 (χ2)

Figure 4.2 – SP-IR is rarely observed in ovarian cancer or normal ovary. (A) Ovarian serous tumours showing rare SP-IR presumed neuroendocrine cells. Image on the right shows SP-IR cell at higher magnification. (B) Mucinous tumours showing SP-IR cells of similar morphology to that observed in serous. (C) Section of normal ovary also showing SP-IR presumed neuroendocrine cells (white arrow) . (D) Number of cases with no SP-IR (SP -), SP-IR in either nerves or neuroendocrine cells (SP +), or SP-IR in nerves. Scale bar = 50µm. BV= blood vessel

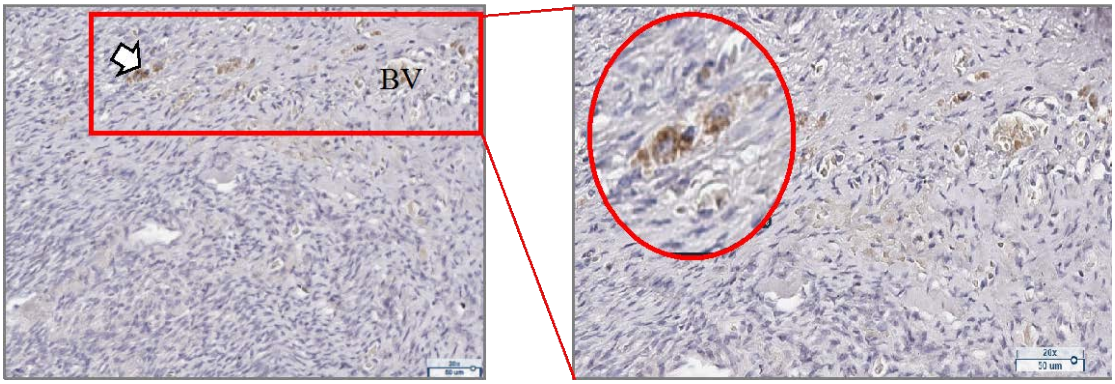
A.1 OvCa Serous



A.1 OvCa Serous



C.1 Normal Ov



D

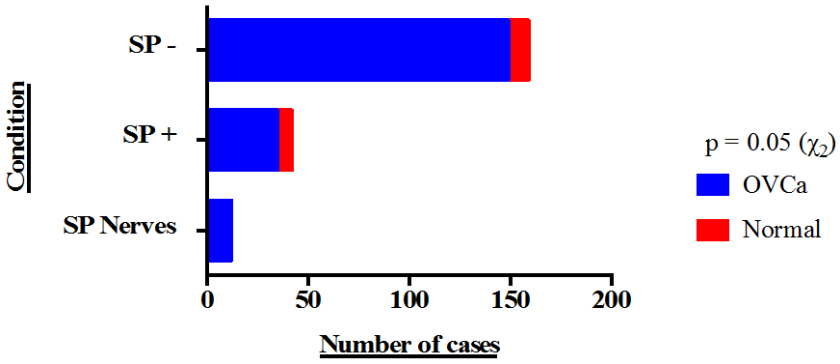
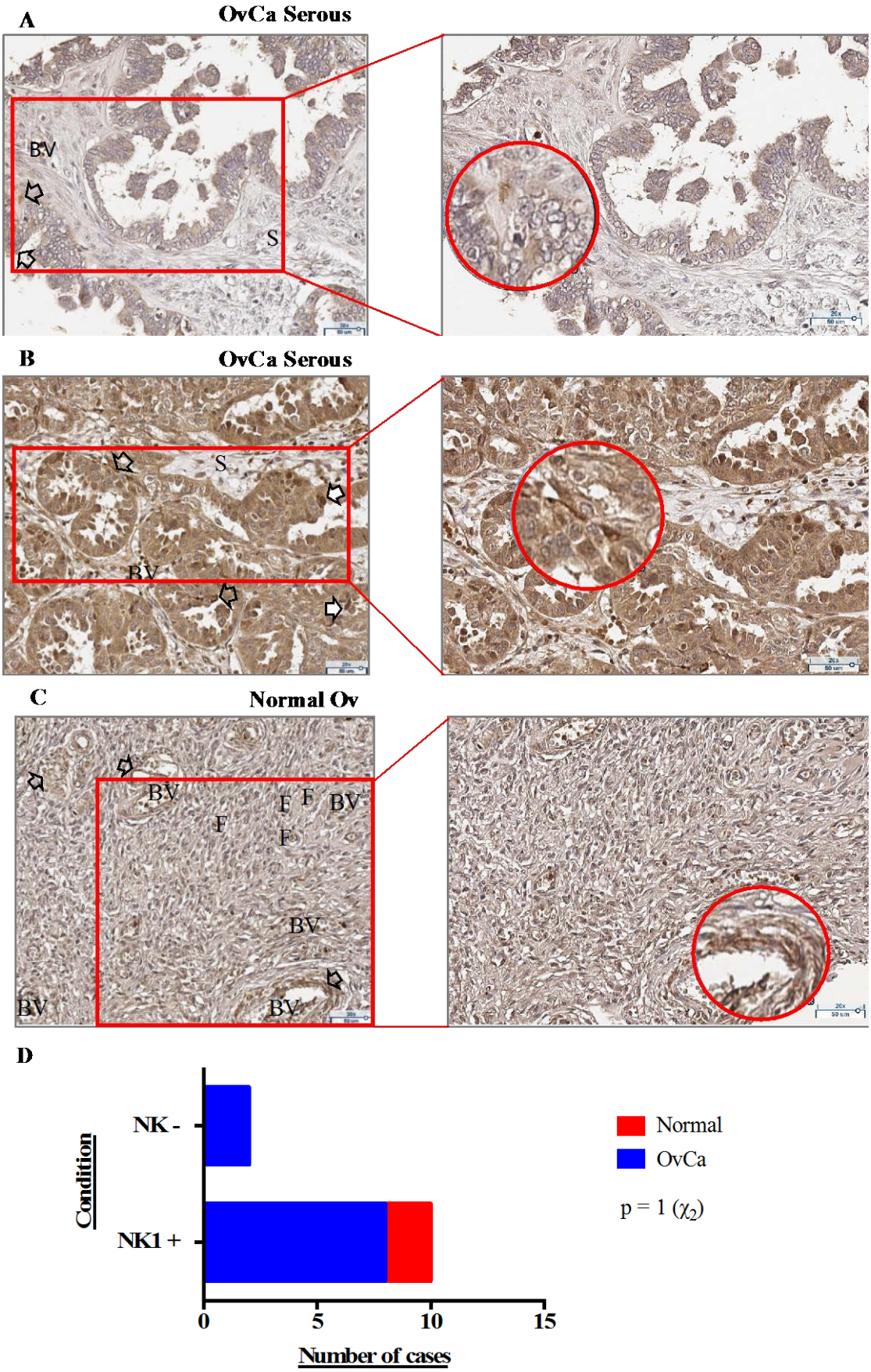


Figure 4.3 – NK1-IR in ovarian cancer and normal ovary. (A) Ovarian serous tumours showing NK1-IR in restricted regions of the stroma. (B) Serous tumours showing wide spread NK1-IR in cancer cells and stroma. (C) Normal ovary showing NK1-IR cells in the stroma and adjacent to blood vessels (BV). White arrow, NK1-IR presumed neuroendocrine cells. Arrow, NK1-IR axons. F, ovarian follicles. (D) Quantification of the number of cases showing no NK1-IR (NK1 -) or positive NK1-IR (NK1 +). Scale bar = 50µm


A
OvCa Serous
BV
S
B
OvCa Serous
S
BV
C
Normal Ov
BV
F
F
BV
F
F
BV
BV
BV
D
Condition
NK -
NK1 +
0
5
10
15
Number of cases
Normal
OvCa
p = 1 (χ2)

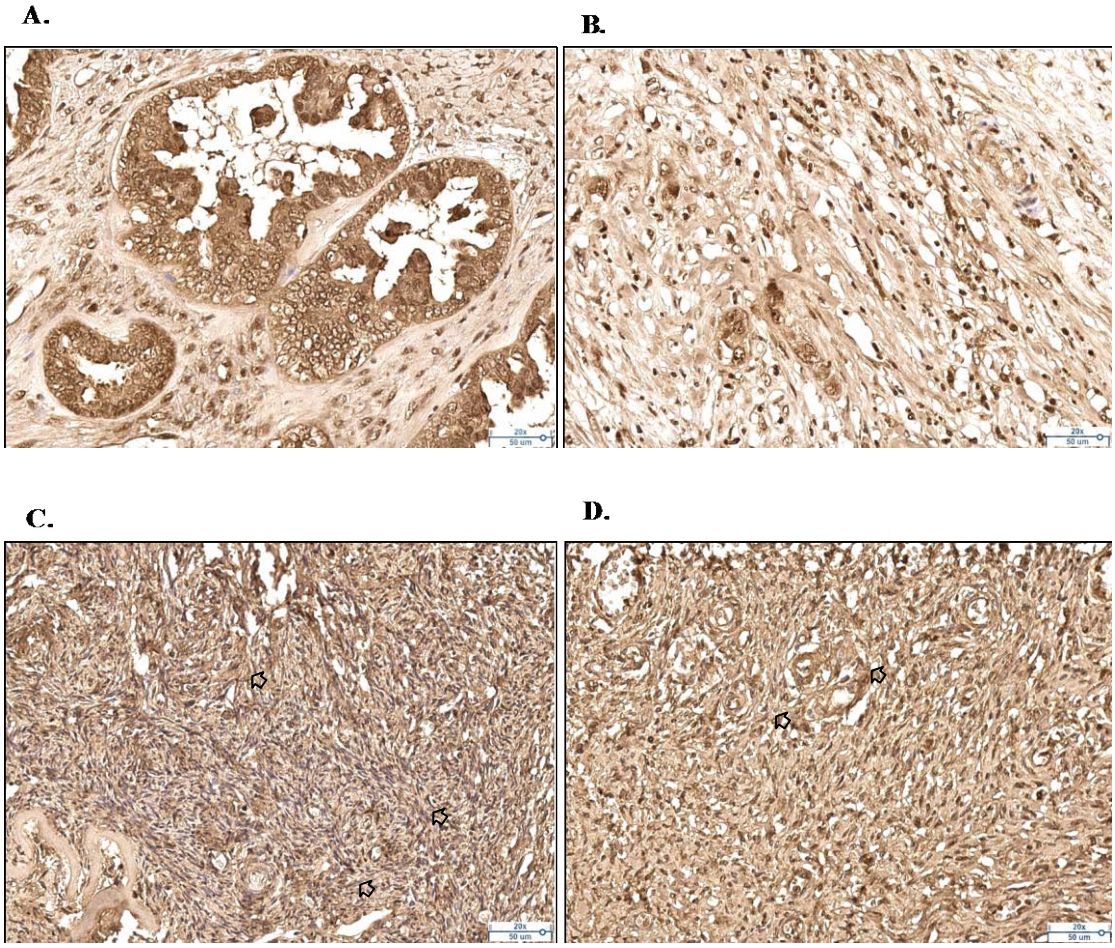


Figure 4.4 – nNOS-IR in ovarian cancer and normal ovary. (A) nNOS-IR expressed in cancer cells and stroma of serous tumours. (B) Mucinous tumour also showing nNOS in cancer cells and throughout the stroma. (C & D) Two normal ovary sections showing nNOS in presumed axons (arrows) but also in other cells. nNOS-IR is not restricted to nerves in either cancer or normal ovary, altough our nNOS antibody was selective for neurons in the small intestine (chapter 2). Our negative control (no primary antisera) showed no immunoreactivity thus validating our method. Scale bar= 50µm

CHAPTER 5: EXPRESSION OF TROPHIC AND GROWTH FACTORS RECEPTORS IN OVARIAN CANCER

5.1. Background

Cells in tumours and normal tissues rely on bidirectional signals mediated by cell surface proteins to maintain their function and homeostasis. It was recently suggested that we should integrate concepts from signalling in normal tissues to the tumour microenvironment to achieve advances in diagnosis and therapies (Venere, Lathia et al. 2013). Reports linking cancer and growth factors date back to the 1980s (Goustin, Leof et al. 1986). Thereafter, increased research in the mechanisms of action of these cell-proliferation-stimulating polypeptides proved their relevance to cancer. Namely, the receptor tyrosine kinase family was pinpointed in tumour growth (Harris 1991). This, includes the Trk and the RET (tyrosine kinase receptor for rearranged during transfection) families of receptors which encompass receptors for neurotrophins and the GDNF-family ligands (GFLs), respectively.

Each Trk receptor has different binding affinity to specific neurotrophins. While $p75^{NTR}$ receptor can bind all neurotrophins, it was reported to have a higher affinity for pro-neurotrophins (Oppenheim, Milligan et al. 2013). Mature neurotrophins reportedly bind preferably with their specific Trk receptor (Oppenheim, Milligan et al. 2013). However, these affinity binding assays were limited to some tissues and the relative affinities of the various receptors may need to be revisited. TrkA receptor binds with high affinity to NGF and TrkB (tyrosine receptor kinase B) to brain-derived neurotrophic factor (BDNF) and NT-4. Both TrkA and TrkB can bind NT-3 but to a

lesser level than TrkC (tyrosine receptor kinase C). TrkC is mostly expressed by proprioceptive sensory neurons and TrkA in peptidergic nociceptive sensory neurons (McMahon, Armanini et al. 1994, Smeyne, Klein et al. 1994, Segal 2003). Concurrently, $p75^{NTR}$ affects Trk receptors specificity and affinity and hence, their activation. I.e., for example, $p75^{NTR}$ expression is important to increase the binding affinity of NGF to TrkA, while it can also reduce ligand-induced receptor ubiquitination and delay receptor internalization and degradation (Friedman and Greene 1999, Segal 2003).

The renewed interest Trk family of receptors relates to descriptions of their role in human cancers (Geldof, Van Haarst et al. 1998, Hondermarck 2012). Molecular characterization of neurotrophins and their receptors along with the identification of oncogenic modifications in the gene for TrkA (NTRK1), TrkB (NTRK2), TrkC (NTRK3) and $p75^{NTR}$ suggest that the TRK oncogene family may represent a therapeutic target (Dalal and Djakiew 1997, Latil and Lidereau 1998, Wessels, Wu et al. 2014, Vaishnavi, Le et al. 2015).

Sortilin is another receptor and/or co-receptor found ubiquitously expressed in many tissues but more prominent in the CNS (Wilson, Naves et al. 2016). $P75^{NTR}$ interaction with TrkA enhances neuron survival, while proNGF binding to $p75^{NTR}$-Sortilin complex initiates neuronal apoptosis (Nykjaer, Lee et al. 2004, Linggi, Burke et al. 2005, Clewes, Fahey et al. 2008, Vaegter, Jansen et al. 2011). The cellular response depends on the receptor complex formed and in the cellular context. Deregulation of sortilin glycoprotein has been implicated in major human diseases including type 2 diabetes (Li, Matye et al. 2016) and cancer (Wilson, Naves et al. 2016).

The GDNF family of ligands (GFLs) includes glial cell line-derived neurotrophic factor (GDNF), artemin (ARTN), neurturin (NRTN) and persephin (PSPN). These factors are heavily involved in the development and function of the nervous system (both central and peripheral). In general, GFLs all signal through the receptor tyrosine kinase RET. Their specificity is implemented by different GDNF family receptor α (GFRα), which act as co-receptor. GFRα1-4 are responsible for the binding of GDNF, NRTN, ARTN, and PSPN, respectively, and the subsequent activation of RET. This family of ligands and/or receptors have also been implicated in cancer, namely in thyroid (Severskaia, Saenko et al. 2006), breast (Sakamoto, Kitajima et al. 2001, Esseghir, Todd et al. 2007) and ovarian tumorigenesis (Aravindakshan, Chen et al. 2006).

Our group has identified and described the role of neurotrophins and their receptors (including proNGF, sortilin and $p75^{NTR}$) across prostate , breast (Demont, Corbet et al. 2012, Roselli, Pundavela et al. 2015) and thyroid (Faulkner, Roselli et al. 2016) cancers.

In this chapter, I aimed to identify the most prominent receptors for neurotrophic factors present in the ovarian tumour microenvironment. Because autonomic and sensory neurons of the peripheral nervous system require target-derived neurotrophins for their survival and function, experiments were conducted to investigate if some of these receptors, namely TrkA, TrkB, $p75^{NTR}$ and sortilin, were expressed in cells and/or fibres in human adult ovarian tissue microarrays from normal and cancers biopsies. Other prospective growth factors present in the ovarian tumour microenvironment are the GDNF family. However, before checking for GDNF presence it is important to assess if the necessary receptors for its functions are present in the ovarian tumour cells and/or tumour microenvironment. Thus, due to the known role of the GDNF family of

ligands in establishing pelvic innervation, I tested the expression of GFRα1, 2 & 3 receptors.

5.2. Materials And Methods

Protocols were as outlined in chapter 2.

5.2.1. Ovary tissue samples

Tumour microarrays (TMAs) of ovarian cancers and adjacent normal and normal ovarian tissue (T112b and OV20810) were obtained from US Biomax, Inc. (Maryland, USA). This cohort included 158 serous adenocarcinomas and 35 mucinous adenocarcinomas. In addition, 2 clear cell carcinomas, 2 endometrioid, 1 granular and 4 adenocarcinomas of unknown type were also included.

5.2.2. Immunohistochemistry

After deparaffinization and hydration of tumour microarrays as per (chapter 2), Slides were probed with anti-TrkA 1:200 (2508S), anti-TrkB 1:1000 (ANT-019), anti-$p75^{NTR}$ 1:400 (4201S), anti-Sortilin 1:1000 (ANT-009), and anti-GFRα1, 2 & 3 (AF560, AF429 and AF2445, respectively), or normal IgG, respectively (Alpha Diagnostic) as a negative control. Image capture and analysis was as outlined in chapter 2 as was statistical analysis.

5.3. Results

5.3.1. TrkA receptor expression is a feature of ovarian cancer but is not necessarily linked to that of NGF

The expression of high affinity NGF receptor TrkA in ovarian cancer was investigated. TrkA-IR was observed in immune-like cells, in neuroendoctine -like cells and/or axons. TrkA-IR was also observed in the cytoplasm and membranes of cancer cells and in the ovarian tumour stroma (Figure 5.1).

TrkA-IR was relatively weak in most samples. The strongest immunoreactivity was observed in specific groups of cells in the tumour stroma and often in a subset of cells that lay between the tumour stroma and cancer cells. This is most apparent in cases of mucinous ovarian carcinoma (Figure 5.1 B). In serous tumours TrkA-IR was often observed in the stroma in moderate intensity (Fig 5.1 A). In normal ovary TrkA-IR was scarce with scattered TrkA-IR cells of relative low intensity and immune-cell morphology (i.e. Light brown opposite to the dark brown in the mucinous tumour cancer/stromal cells) (Fig 5.1 C). The presence or absence of TrkA-IR was not statistically significant different between normal and ovarian cancer samples overall ($p=0.5328$). More interestingly no significant link was found to NGF-IR ($p=0.1607$; Table 5.1). However, mucinous ovarian tumours showed proportionally more TrkA-IR than the serous types ($p=0.0124$; Table 5.1).

No other statistical significance to clinicopathological parameters was established.

5.3.2. TrkB expression is substantial in ovarian cancer

TrkB is a receptor tyrosine kinase of the Trk family. TrkB is a receptor for brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4/5 but not NGF. The involvement of TrkB in ovarian folliculogenesis and ovarian cancer tumorigenesis was previously suggested and thus we looked to study its expression in 12 ovarian tumours. All the samples investigated were positive for TrkB-IR (Table 5.2). TrkB was preferably observed in the membrane of cancer cells and in stromal cells (Figure 5.2). Interestingly, not all cancer cells within the same tumour and/or tissue microarray showed TrkB-IR suggesting specificity of this receptor to cancer associated-cell populations. Also noteworthy is that normal ovary showed mid-intensity TrkB-IR with most of the stain in the granular cells of ovarian follicles and in the membranes of the blood vessels (Figure 5.2 C). TrkB-IR axons were also detected in the ovarian stroma, particularly near follicles and blood vessels.

5.3.3. Ovarian cancer expresses $P75^{NTR}$

The neurotrophin transmembrane receptor $p75^{NTR}$ was also investigated due to its low-affinity binding to NGF, BDNF, NT-3 and NT-4. $P75^{NTR}$-IR was studied across a cohort of 202 ovarian tumours and 18 normal ovarian tissue microarrays. Area of expression varied across ovarian tumours but is expressed throughout all samples analysed. Specific cells of small size showed particularly high intensity of $p75^{NTR}$-IR in ovarian tumours (Figure 5.3 A and B). In the normal ovary, small cells also showed high $p75^{NTR}$ expression but in much lower numbers). In addition, most of the $p75^{NTR}$ immunostaining appears near blood vessel in the stroma of the normal ovary (Figure 5.3 C).

5.3.4. Sortilin expression is a potential feature in high-grade serous and/ or rare ovarian tumours

Sortilin is known for binding pro-apoptotic precursor forms of BDNF (proBDNF) and NGF (proNGF). Therefore, and with previously having looked at proNGF and other neurotrophins receptors expression, we immunoassayed a small cohort of ovarian cancer samples with sortilin antiserum. Sortilin-IR was observed less frequently than that observed for $p75^{NTR}$ and TrkB receptors (Figure 5.4). In our small sample of 10 cancers sortilin-IR was observed only in the intima layer of some blood vessels in 3 cases: one endometrioid, one clear cell and one high-grade serous tumour (Figure 5.4 C; Table 5.3). Sortilin-IR in these samples was observed mostly in the cytoplasm of very specific cancer cells (including apoptotic cancer cells) and some cancer-cell-associated fibroblasts. No sortilin-IR was detected in stroma cells of either cancer or normal tissue. Plus, only a couple of mucinous ovarian tumour cells were found positive for sortilin (Figure 5.4 B, Table 5.3). In this small cohort, it was not possible to establish any correlation between sortilin expression and clinopathological parameters. However, investigation of a larger cohort will need to be performed to test if sortilin expression is linked to higher grade ovarian cancer.

5.3.5. GDNF family receptor alpha 1, alpha 2 and alpha 3 are expressed in normal and cancer ovary

Specific co-receptors for GDNF, NRTN and ARTN, $GFR_{\alpha 1\text{-}3}$ respectively, were investigated in a small cohort of ovarian tumours. The three receptors were found to individually localize to specific cells and/or structures in parallel to being expressed in common regions (Figure 5.5, Figure 5.6). GDNF receptor $GFR\alpha_1$ was observed in

blood vessels namely, in the adventitia layer and in oocytes of primordial and primary follicles of the normal human ovary. Later-stage follicles showed some GFRα_1-IR but fainter. GFRα_1 staining was also present in many somatic cell types in normal human ovary (Figure 5.5 C). Many somatic cells in the adult ovarian stroma also showed GFRα_3 -IR followed, to a lesser extent, by GFRα_2-IR (Figure 5.6 A and Figure 5.7 C). GFRα_2 staining was observed specifically in the early human ovarian follicles (Figure 5.6 C). In parallel, GFRα_3 –IR showed a pattern more similar to GFRα_1-immunostaining with granular and oocyte staining in the primordial and primary follicles, plus also some in later follicles (Figure 5.7 C *vs*. Figure 5.5 C).

In ovarian tumours, GFRα1 was localised to cancer cells cytoplasm and in tumour-associated cells, seemingly fibroblasts and/or a few axons (Figure 5.5 A). This immunostaining showed intensity variation across ovarian tumours (Figure 5.5. A). Plus, mucinous ovarian tumours showed faint GFRα1-IR except for some specific cancer associated cells (Figure 5.5 B).

Identical to GFRα1-IR, mucinous ovarian tumours showed GFRα2-IR in some fibres and/or axon like structures across the tumour and nearby blood vessels, and in cancer-associated cells, especially basal to the mucinous tumour cells (Figure 5.6 B). In ovarian serous tumours, also as with GFRα1 staining, GFRα2 expression varied across tumours (Figure 5.6 A). While some serous cancer cells showed high cytoplasmic expression of GFRα1-IR, others showed varicose-like GFRα2-positive fibres (A). Plus, higher-grade serous tumours (A, bottom) showed GFRα2 expression in some epithelial elongated somatic cells in the tumours stroma, hence slightly different from GFRα1-IR. Note that blood cells did not stain with GFRα1 or GFRα2 antisera.

GFRα3 showed the highest protein expression observed amongst the receptors of the GDNF family investigated. It was localised to ovarian cancer cells, specifically within the cells' basal membrane and neighbouring the tumours' stroma (Figure 5.7 A and B). Some somatic cells in the tumours stroma were also GFRα3-IR positive. Likewise, GFRα3 staining intensity showed variances between ovarian serous carcinomas (Figure 5.7 A); serous low-grade followed by high-grade serous ovarian tumours).

5.4. Discussion

Potential links between neurotrophic factors and cancer aetiology and/or metastasis have been discussed (Demir, Friess et al. 2012, Bradshaw, Pundavela et al. 2014). The ovary of humans and other mammals is innervated by sympathetic and sensory neurons of the peripheral nervous system. The normal development is known to be under strict and step-wise control of growth factors and its receptors. However, their role in disease, namely in cancer pathologies, seems to be more complex. Targeting growth factors has been proposed as a potential therapeutic strategy (Pundavela, Demont et al. 2014, Jobling, Pundavela et al. 2015, Pundavela, Roselli et al. 2015).

NGF was the best known neuronal growth factor and its high-affinity receptor TrkA were analysed in oesophageal (Zhu, Friess et al. 2000), breast (Descamps, Toillon et al. 2001, Lagadec, Meignan et al. 2009), lung (Ricci, Greco et al. 2001), thyroid (Greco, Miranda et al. 2010), hepatic (Kishibe, Yamada et al. 2002) and ovarian (Tapia, Gabler et al. 2011) cancers Thus, suggesting a potential paracrine/autocrine regulation

in the development of non-neuronal carcinomas via stromal/tumoural NGF-TrkA interactions (Koizumi, Morita et al. 1998, Demir, Tieftrunk et al. 2016). At this stage, these receptors were discussed in the context of angiogenesis and not neurogenesis. NGF and TrkA protein expressions were found in endothelial cells of effusions and solid ovarian tumours. Furthermore, TrkA in effusions seemed to predict better outcome whereas membrane expression of TrkA in solid tumours were correlated with poor outcome in advanced stage serous ovarian carcinoma (Davidson, Reich et al. 2003). Identical observations were made in breast tumours suggesting that the expression of NGF and TrkA in primary tumours may be linked to tumour aggression in both breast and epithelial ovarian cancers (Davidson, Reich et al. 2004, Campos, Munoz et al. 2007). We too observed high expression of TrkA in ovarian cancer cells, but even more so in mucinous ovarian carcinomas over serous tumours. TrkA was not particularly expressed in endothelial cells, yet being highly expressed in the stroma of some epithelial serous and mucinous ovarian tumours. TrkA-IR was not significantly related to clinicopathological parameters either, and connections to patient outcome could not be established.

We could not confirm previous reports associating TrkA protein and gene expression with higher-grade and more aggressive ovarian carcinomas (Koizumi, Morita et al. 1998, Davidson, Reich et al. 2003, Davidson, Reich et al. 2004, Odegaard, Staff et al. 2007, Tapia, Gabler et al. 2011). Moreover, we found little expression of TrkA in the normal human adult ovary. The reported levels of TrkA in normal ovary vary amongst studies (Dissen, Hill et al. 1996, Mayerhofer, Dissen et al. 1996, Koizumi, Morita et al. 1998, Shi, Jin et al. 2004, Salas, Julio-Pieper et al. 2006, Tapia, Gabler et al. 2011, Vera, Tapia et al. 2014) and may relate to the particular ovulatory status of

our ovarian tissue microarrays. It was previously suggested that the TrkA gene activation occurs in a remarkably narrow time frame, namely at the end of follicular growth. (Dissen, Hill et al. 1996, Mayerhofer, Dissen et al. 1996).

Neurotrophins other than NGF have also been highlighted in the regulation of the human oocyte maturation and ovarian function (Spencer, Waters et al. 2010, Zhao, Qiao et al. 2011). BDNF and its high-affinity receptor TrkB have been scantily described in colorectal (Tanaka, Okugawa et al. 2014), ovarian (Au, Siu et al. 2009), uteri (Moon, Won et al. 2011) and breast (Vanhecke, Adriaenssens et al. 2011) cancers. Additionally, BDNF has been mechanistically described to promote chronic stress effects on ovarian tumour progression (Allen 2012). We found BDNF receptor, TrkB, to be highly expressed in ovarian tumours, although also expressed in the cytoplasm of normal ovarian stroma cells. TrkB protein was overexpressed in ovarian cancer cells and varied slightly amongst tumours. Differences in TrkB presence/absence between tumour types could not be established, but serous tumours stroma cells showed low to nil expression while stroma cells in mucinous carcinomas showed strong TrkB-expression. The pathophysiological significance of this requires further investigation. It is known that BDNF acts through TrkB during the development of the neonatal ovary and assures the survival of central and peripheral neuronal populations (Jensen and Johnson 2001, Pezet, Malcangio et al. 2002, Paredes, Romero et al. 2004, Slack, Grist et al. 2005, Dorfman, Kerr et al. 2011). Additionally, BDNF/TrkB were shown to exacerbate ovarian cancer migration and invasion while inhibiting apoptosis hence correlating to shorter patient survival (Au, Siu et al. 2009, Siu, Wong et al. 2009). Thus, BDNF/TrkB pathways need to be further explored. TrkB can also interact with NT-4/5 and to a lesser extent NT-3. This should be considered in further studies.

Other neurotrophic factor receptors have been identified and described in the ovarian tumour microenvironment, namely, the low-affinity receptors $p75^{NTR}$ and sortilin.

In the 1990s, $p75^{NTR}$-IR in glial cells were described to associate with TH-IR outgrown axons after peripheral nerve injury (Zhou, Rush et al. 1996). However, soon after $p75^{NTR}$ was reported to mediate neuronal death (Bamji, Majdan et al. 1998).

We found $p75^{NTR}$ to be universally expressed across all tissue microarrays, including in the normal adult ovary. As previously reported in non-human primate ovaries, $p75^{NTR}$ expression was observed in vasculature, interstitial tissues and in growing follicles as well as in neuroendocrine cells of adult human normal ovary (Dees, Hiney et al. 1995). Notably, the ovary has been reported to synthesise neurotrophin receptors such as $p75^{NTR}$ keeping its expression high to regulate ovarian function and command follicles assembly (Dees, Hiney et al. 1995, Dissen, Hirshfield et al. 1995). Similar cues may be at play during disease but orchestrated by tumour cells requirements. $P75^{NTR}$ protein expression showed variable area and intensity of expression in ovarian tumours.

$p75^{NTR}$ was reported to induce neuronal apoptosis when activated in the absence of Trk signal, while in the presence of Trk it enhanced neurotrophic response (Kaplan and Miller 1997). Moreover, absence of $p75^{NTR}$ blocks the development of more than one neuronal subtype (Bergmann, Priestley et al. 1997).

As opposed to $p75^{NTR}$ activation, when sortilin/Trka and proNGF interact, breast cancer cells were found to become more invasive (Demont, Corbet et al. 2012). Sortilin interacts with TrkA, TrkB and TrkC to allow anterograde axonal transport consequently enhancing neurotrophins signalling. Furthermore, sortilin also modulates neuronal

survival and aggravates TrkA, TrkB and TrkC phenotypes in the absence of $p75^{NTR}$, leading to embryonic lethality and sympathetic neuropathy in TrkA heterozygote mice (Vaegter, Jansen et al. 2011). However, TrkA has been implicated as pro-apoptotic when linked to pro-neurotrophins (Hemmati, Zarnani et al. 2009, Teng, Felice et al. 2010).

The importance of neurotrophins for cancer progression is likely to depend on the relative expression of their receptors. For example, in the cases where $p75^{NTR}$ and TrkA are both present, NGF would be expected to drive tumour progression. Where $p75^{NTR}$ is expressed without TrkA, NGF will have little effect but exposure to proNGF and sortilin may drive apoptosis (Bradshaw, Pundavela et al. 2014). The consequences of TRkB expression will depend on the presence of BDNF. I found wide expression of TrkB, which suggests that BNDF needs to be considered for ovarian cancer cell progression.

The GDNF family of growth factors has been mainly described for axonal growth and development in the GIT but more recently in pelvic visceral innervation (Forrest and Keast 2008, Allen, Watson et al. 2013, Forrest, Osborne et al. 2014). In this work, I found all the GDNF family receptors, GFRα1-3, were present in the normal human adult ovary namely in endothelial and follicular/oocyte cells. This is the first description of these receptors in the ovary and warrants further investigation. GDNF studies in murine models, including links to ovarian tumorigenesis in age-dependent models, further support this (Golden, DeMaro et al. 1999, Aravindakshan, Chen et al. 2006, Zhao, Qiao et al. 2011). Furthermore, the implications of GDNF family ligands and receptors for neuronal development and recently-developed target drugs against them

for CNS diseases, such as Parkinson's disease, further suggest a dual-interaction between ovarian cancers and peripheral nervous system (Baloh, Enomoto et al. 2000, Bespalov and Saarma 2007, Allen, Watson et al. 2013). A recent publication in pancreatic cancer cells lines highlights the role of GFRα1 released by nerves into inducing perineural invasion whilst tissues microarray of human pancreatic ductal adenocarcinomas showed wide variance of cancer cell GFRα1 expression (He, Chen et al. 2014). This too warrants further investigation.

Table 5.1 - Association between the presence of TrkA and clinicopathological parameters of ovarian tumours. Whilst TrkA expression in cancer samples was only slightly higher than in histologically normal ovarian tissue. Mucinous carcinomas showed significantly more TrkA than Serous carcinomas (p=0.0124). Within the mucinous tumours, no relation to TrkA expression could be established with the tumour size (TNM grading, T3= Tumor invades through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues – see chapter 2, table 2.3 *) due to lack of samples. Lower grade tumours (serous and mucinous) show slightly higher expression of TrkA than higher grade (p=0.1883), a trend also slightly observed with NGF expression (chapter 3, table 3.2). Morevoer, some more NGF-expressing axons were observed in TrkA expressing carcinomas (p=0.1607).

PARAMETER	TRKA NEGATIVE	TRKA POSITIVE	P-VALUE
All cases (n=220)			
Normal (n=18)	11 (61%)	7 (39%)	0.5328
Cancer (n=202)	108 (53%)	94 (47%)	
Pathological subtype			
Serous (n=158)	91 (58%)	67 (42%)	**0.0124**
Mucinous (n=35)	12 (34%)	23 (66%)	
Clinical parameters of tumours			
Patient Age			
≤ 50 (n=97)	50 (52%)	47 (48%)	0.5992
> 50 (n=105)	58 (55%)	47 (45%)	
Tumour size (T)			
Serous			
T<3 (n=138)	79 (57%)	59 (43%)	0.8159
T>3 (n=20)	12 (60%)	8 (40%)	
Mucinous			
T<3 (n=35)	12 (34%)	23 (66%)	-
T>3 (n=0)	-	-	
Tumour grade			
Low (grade 1; n=67)	31 (46%)	36 (54%)	0.1883
High (grades 2-3; n=119)	67 (56%)	52 (44%)	
Lymph Node Invasion (N)			
Negative (n=183)	96 (52%)	87 (48%)	0.3735
Positive (n=19)	12 (63%)	7 (37%)	
NGF			
Nerves Negative	82 (57%)	63 (43%)	0.1607
Nerves Positive	26 (46%)	31 (54%)	

Table 5.2 - Association between the presence of TrkB and clinicopathological parameters of ovarian tumours. No significant conclusions can be draw from the

expression of TrkB in ovarian carcinomas since there was extensive immunoreactivity in both normal and tumour samples and we had a small population of samples. Not all cells in the cores expressed TrkB validating the antisera specificity.

PARAMETER	TRKB NEGATIVE	TRKB POSITIVE
All cases (n=12)		
Normal (n=2)	0	2 (100%)
Cancer (n=10)	0	10 (100%)
Pathological subtype		
Serous (n=3)	0	3 (100%)
Mucinous (n=2)	0	2 (100%)
Clear Cell (n=2)	0	2 (100%)
Endometrioid (n=2)	0	2 (100%)
Granular cell (n=1)	0	1 (100%)
Clinical parameters of tumours		
Patient Age		
≤ 50 (n=9)	0	9 (100%)
> 50 (n=1)	0	1 (100%)
Tumour size (T)		
Serous		
T<3 (n=3)	0	3 (100%)
T>3 (n=0)	-	-
Mucinous		
T<3 (n=2)	0	2 (100%)
T>3 (n=0)	-	-
Tumour grade		
Low (grade 1; n=4)	0	4 (100%)
High (grades 2-3; n=3)	0	3 (100%)
Lymph Node Invasion (N)		
Negative (n=9)	0	9 (100%)
Positive (n=1)	0	1 (100%)

Table 5.3 - Association between the presence of Sortilin and clinicopathological parameters of ovarian tumours. As with TrkB, no significant conclusions can be draw from the expression of sortilin in ovarian carcinomas. Sortilin-IR varied across TMAs of the same slide. Most ovarian cancer TMAs did not express sortilin (7 / 10).

PARAMETER	SORTILIN NEGATIVE	SORTILIN POSITIVE
All cases (n=12)		
Normal (n=2)	1 (50%)	1 (50%)
Cancer (n=10)	7 (70%)	3 (30%)
Pathological subtype		
Serous (n=3)	2 (67%)	1 (33%)
Mucinous (n=2)	2 (100%)	0
Clear Cell (n=2)	1 (50%)	1 (50%)
Endometrioid (n=2)	1 (50%)	1 (50%)
Granular cell (n=1)	0	1 (100%)
Clinical parameters of tumours		
Patient Age		
≤ 50 (n=9)	6 (67%)	3 (33%)
> 50 (n=1)	1 (100%)	0
Tumour size (T)		
Serous		
T<3 (n=3)	2 (67%)	1 (33%)
T>3 (n=0)	-	-
Mucinous		
T<3 (n=2)	2 (100%)	0
T>3 (n=0)	-	-
Tumour grade		
Low (grade 1; n=4)	4 (100%)	0
High (grades 2-3; n=3)	1 (33%)	2 (67%)
Lymph Node Invasion (N)		
Negative (n=9)	7 (78%)	2 (22%)
Positive (n=1)	0	1 (100%)

Figure 5.1 –TrkA-IR in ovarian cancer and normal ovary. (A) Serous ovarian tumour showing TrkA localize to the stroma (s), largely absent in cancer cells (cc). (B) Mucinous tumour showing TrkA-IR localize to the border between cancer cells and stroma. (C) Mucimous tumour from another patient showing TrkA-IR surrounding an island of cancer cells. (D) TrkA-IR absent from normal ovary. Images on the right are magnifications of the ones showed in the left panel. bv, blood vessel. Scale bar = 100 µm in left column and Scale bar = 50 µm in right column.

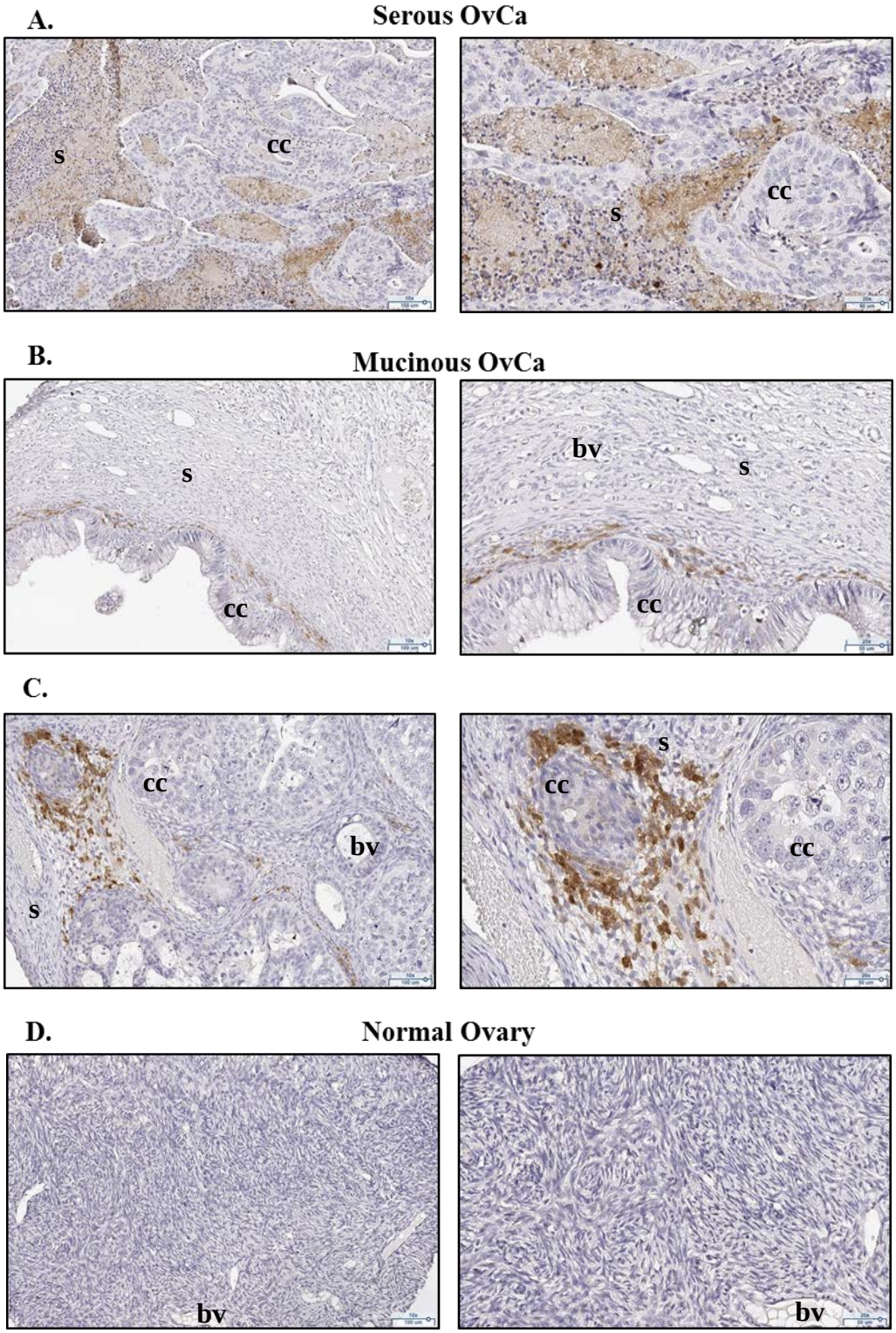
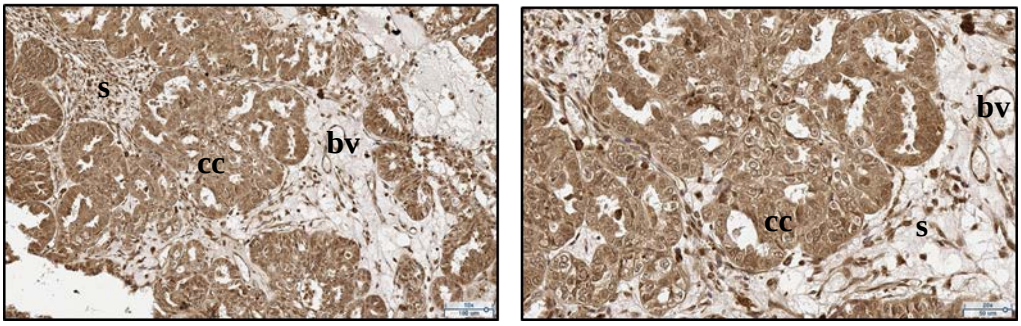

A.
Serous OvCa
s
cc
s
cc
B.
Mucinous OvCa
s
cc
bv
s
cc
C.
cc
bv
s
s
cc
cc
D.
Normal Ovary
bv
bv

Figure 5.2 – TrkB-IR in ovarian cancer and normal ovary . (A) Serous ovarian tumour showing TrkB localize to cancer cells (cc) and stroma (s) cells. (B) Serous tumour from another patient showing TrkB-IR mostly in cancer cells. (C) Mucimous tumour showing wide expressed TrkB-IR (D) TrkB-IR localized to the surrounds of ovarian follicles (f) and blood vessels (bv). Images on the right are magnifications of the ones showed in the left panel. Scale bar = 100 µm in left column and Scale bar = 50 µm in right column.

A. **Serous OvCa**



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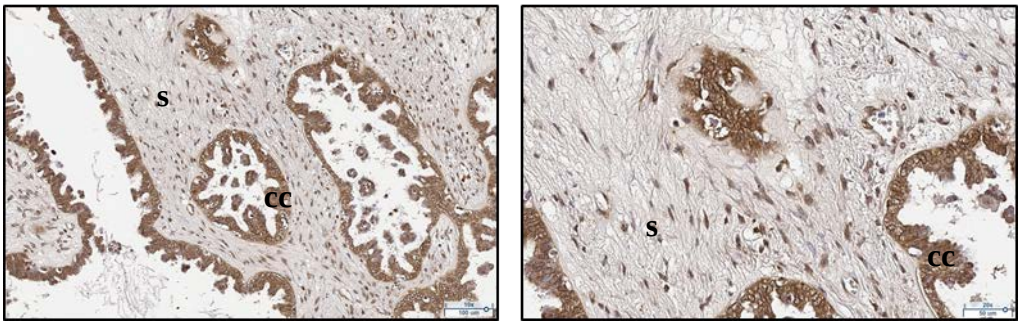
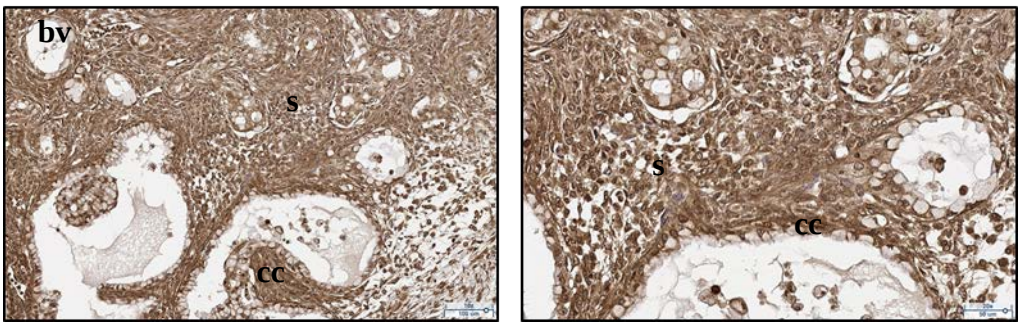


C. **Mucinous OvCa**



D. **Normal Ovary**

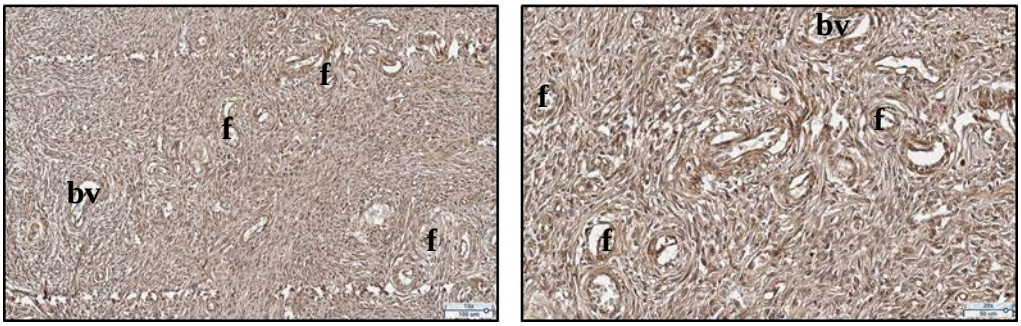
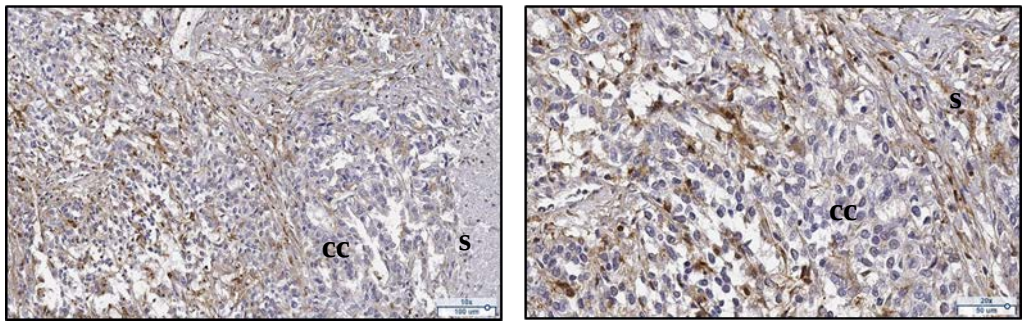


Figure 5.3 – p75^{NTR}-IR in ovarian cancer and normal ovary. (A) Serous ovarian tumour showing p75^{NTR}-IR localized to small cells and putative axons. (B) Serous tumour of another patient showing more intense labelling of p75^{NTR} in cancer cells(cc) and stroma (s). (C) Mucinous tumour showing p75^{NTR}-IR in cancer cells and almost absent from the stroma. (D) p75^{NTR}-IR in several cell types including surrounding blood vessels, follicles and in small cells of undertermined function. Images on the right are magnifications of the ones showed in the left panel. Scale bar = 100 µm in left column and Scale bar = 50 µm in right column.

A. **Serous OvCa**



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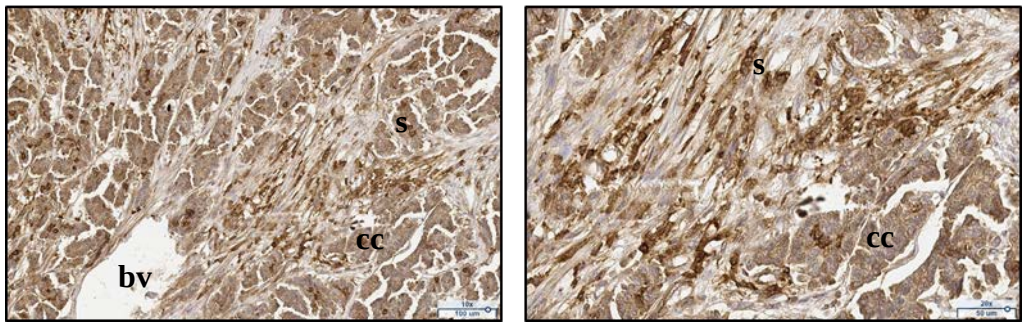
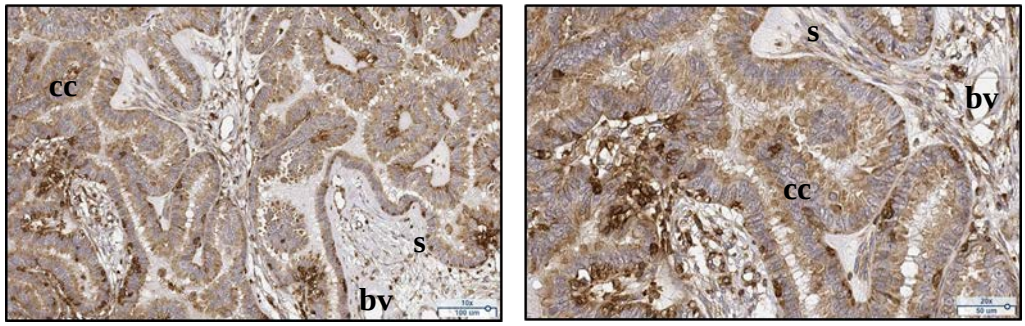


C. **Mucinous OvCa**



D. **Normal Ovary**

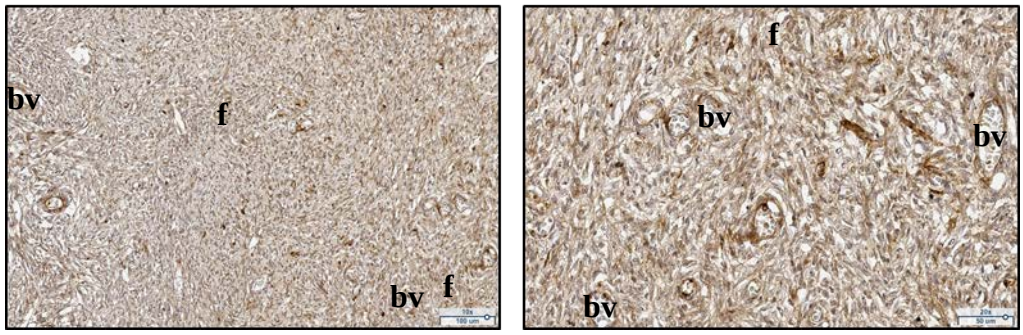
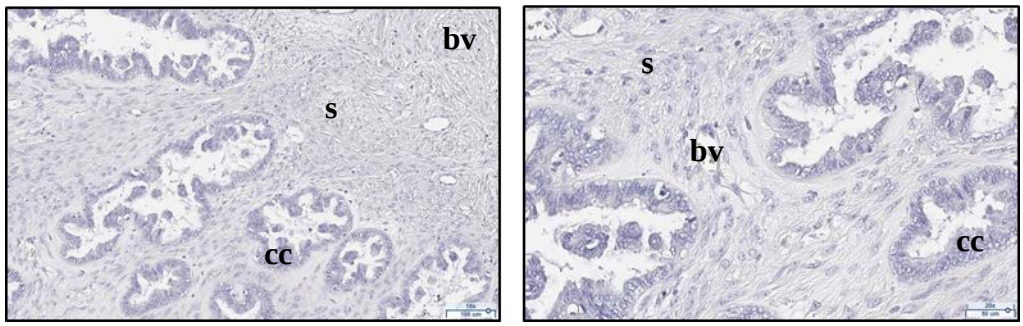


Figure 5.4 – Sortilin-IR in ovarian cancer and normal ovary. (A) Serous ovarian tumour showing absence of sortilin-IR. (B) Serous tumour from another patient showing sortilin-IR in some cancer cells (cc). (C) Mucinous tumours showing only a couple of cells sortilin-IR in the stroma (s). (D) Sortilin-IR absent from the normal ovary. Images on the right are magnifications of the ones showed in the left panel. Scale bar = 100 µm in left column and Scale bar = 50 µm in right column. bv, blood vessel

A. **Serous OvCa**



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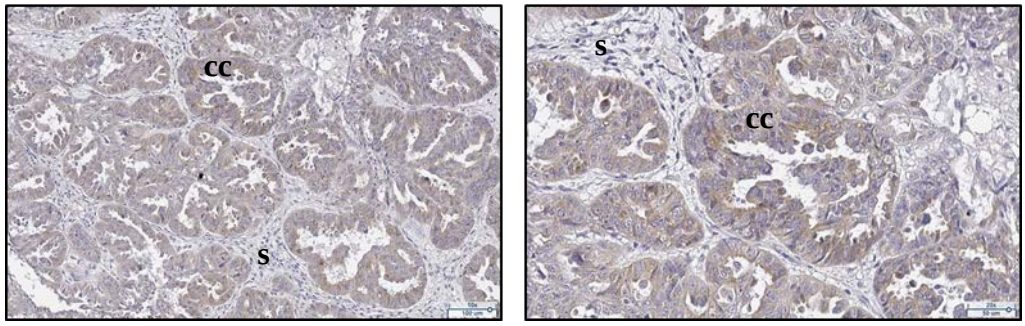
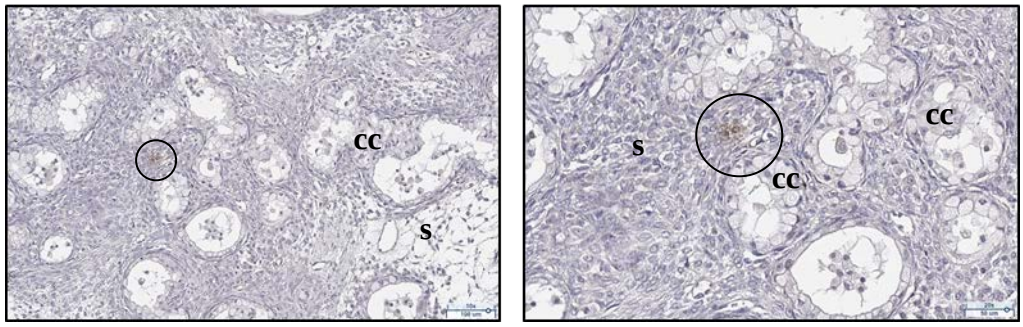


C. **Mucinous OvCa**



D. **Normal Ovary**

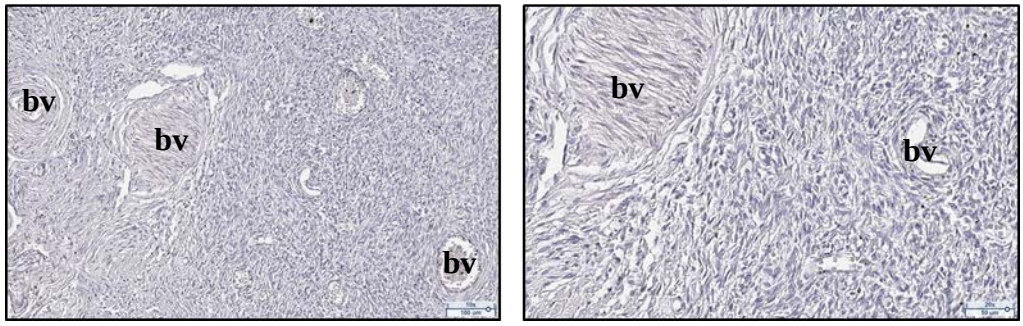
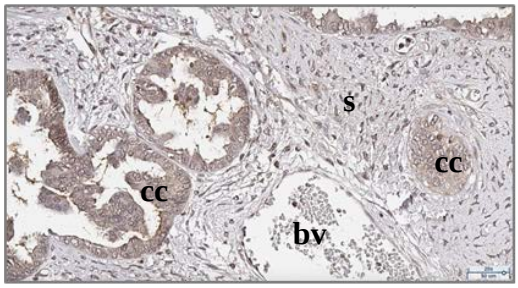


Figure 5.5 - GFRα1-IR in ovarian cancer and normal ovary. (A) Serous ovarian tumour showing GFRα1-IR localize to only a small number of cells. (B) Serous tumour from another patient showing intense labelling of GFRα1 in cancer cells (cc) but only in a few cells in the stroma (s). (C) Mucinous tumours showing GFRα1 labelling in smalls cells in the stroma (cc), cancer cells (cc) and in putative axons. (D) In the normal ovary, putative axons GFRα1-IR surrounding follicles and blood vessels (bv) in sections of two different women. Images on the right are magnifications of the ones showed in the left panel. Scale bar = 100 µm in left column and Scale bar = 50 µm in right column.

A. **Serous OvCa**



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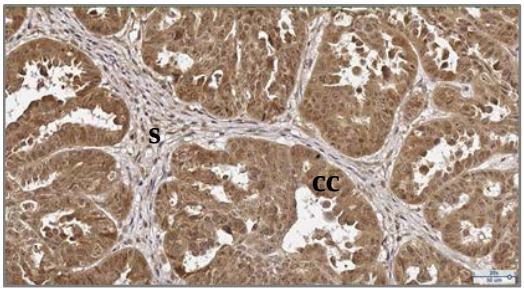
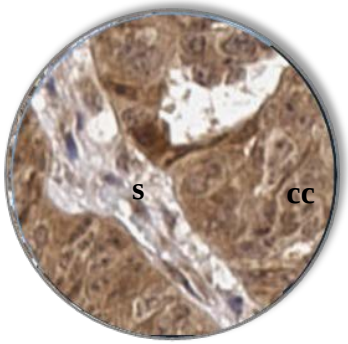
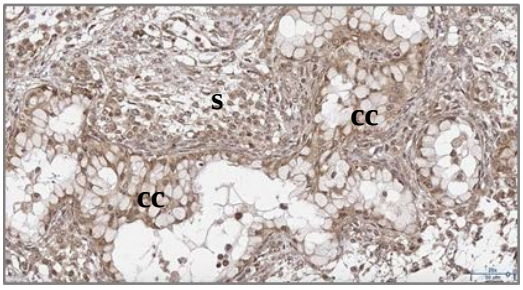
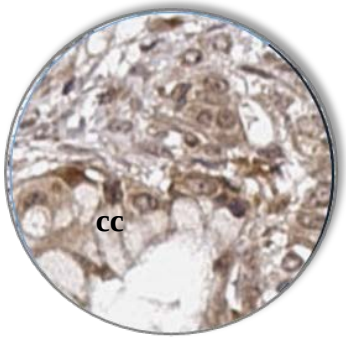


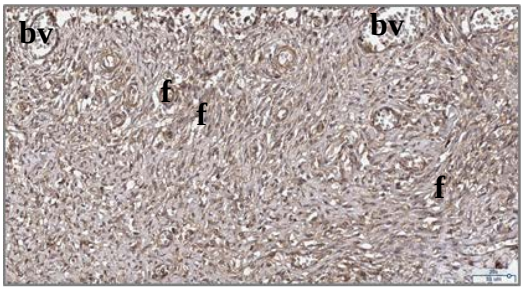


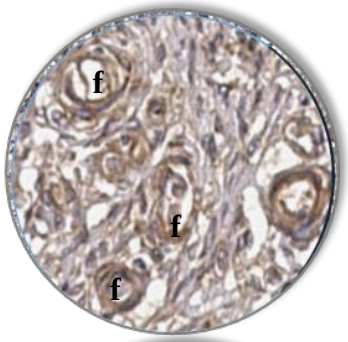
C. **Mucinous OvCa**

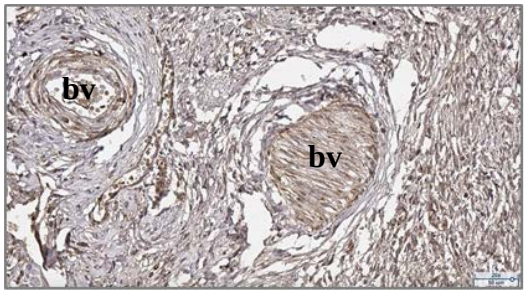




D. **Normal Ovary**







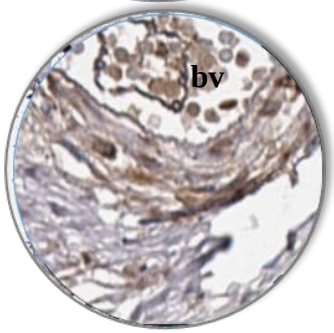
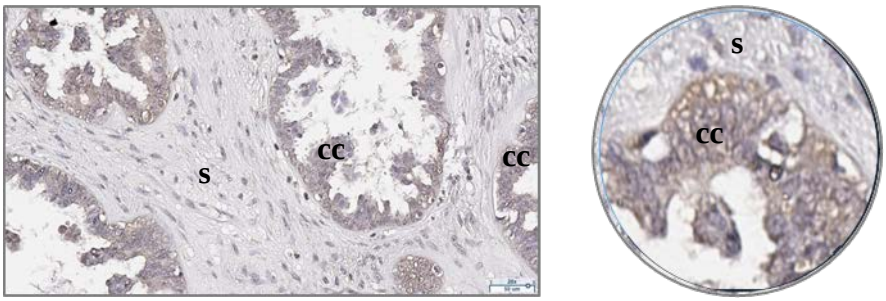
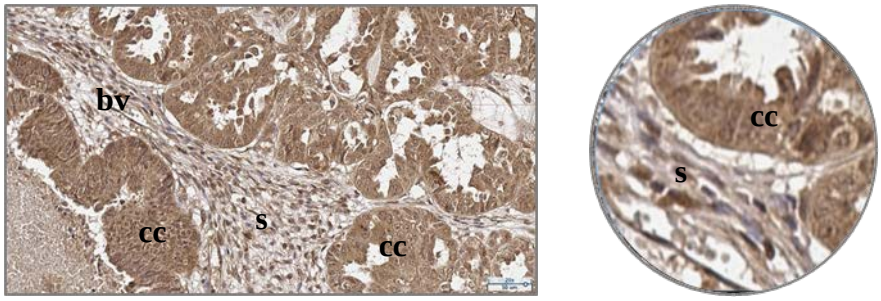


Figure 5.6 - GFRα2-IR in ovarian cancer and in normal ovary. (A) Serous ovarian tumour showing expression of GFRα2 in some cancer cells (cc). (B) Serous tumour from another patient showing intense labelling of GFRα2 in cancer cells (cc) and stroma (s). (C) Mucinous tumours showing GFRα2 labelling in smalls cells in the stroma (cc) and cancer cells (cc). (D) A small number of labelled cells and axons labelled in normal ovary. Images on the right are magnifications of the ones showed in the left panel. Scale bar = 50 µm. bv, blood vessel

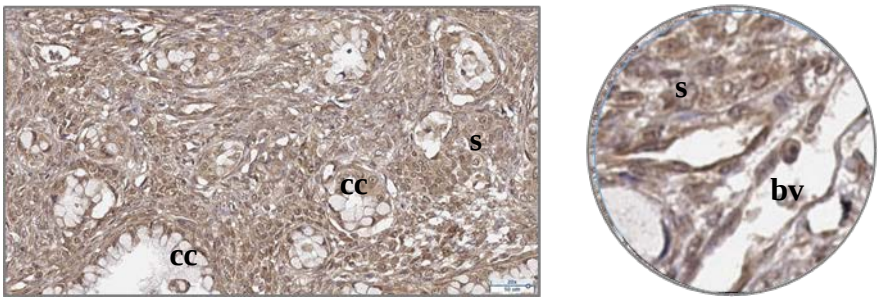
A. **Serous OvCa**



B.



C. **Mucinous OvCa**



D. **Normal Ovary**

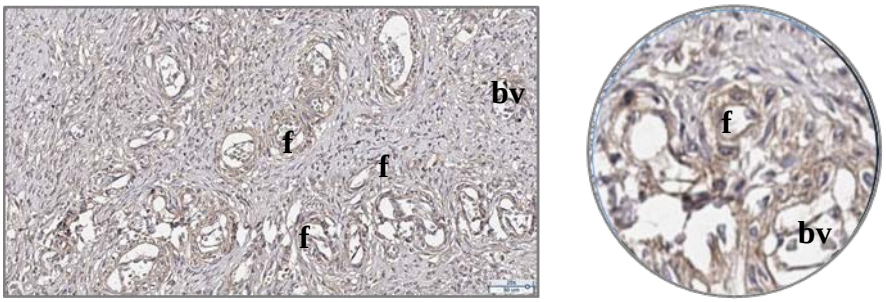
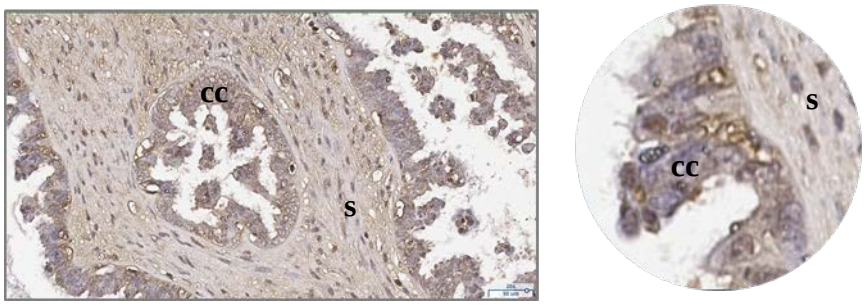
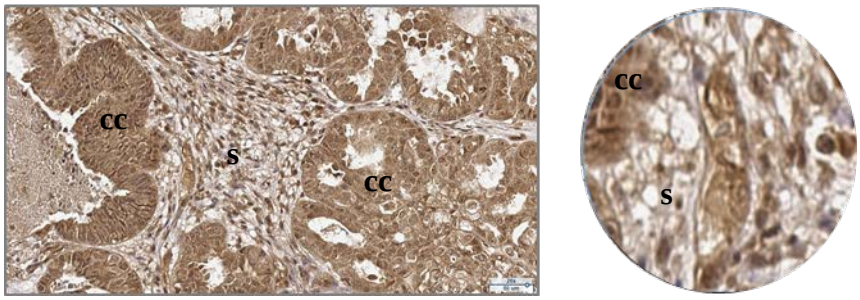


Figure 5.7 - GFRα3-IR in ovarian cancer and in normal ovary. (A) Serous ovarian tumour showing GFRα3-IR putative axons surrounding cancer cells (cc). (B) Serous tumour from another patient showing intense labelling of GFRα3 in cancer cells (cc), small cells in the stroma (s) and putative axons. (C) Mucinous tumours showing GFRα3 labelling in the stroma, cancer cells (cc) and in putative axons. (D) Putative axons GFRα3-IR surrounding follicles, blood vessels (bv) and in cells of unknown function in the normal ovarian stroma. Images on the right are magnifications of the ones showed in the left panel. Scale bar = 50 µm.

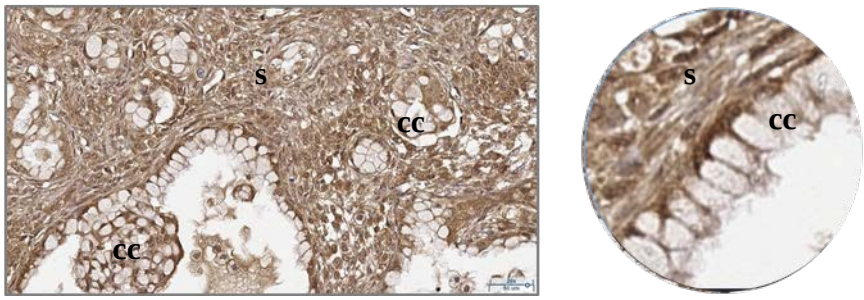
A. **Serous OvCa**



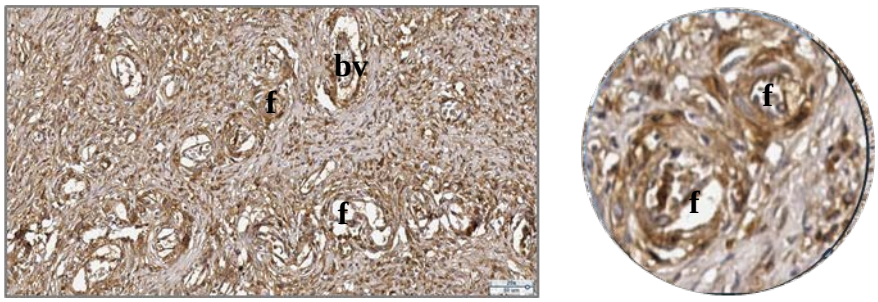
B.



C. **Mucinous OvCa**



D. **Normal Ovary**



CHAPTER 6: OVARIAN CANCER CELLS OVCAR-3 CROSS-TALK WITH NEURONS

6.1. Background

Despite our knowledge of molecular and genetic changes in ovarian cancer we do not understand the mechanism(s) that lead to cancer development, and this is largely due to the lack of a good experimental model system for studying human ovarian cancer (Rosen, Yang et al. 2009).

Human ovarian surface epithelial cells are more difficult to transform than cells from rodents (Orsulic, Li et al. 2002, Zheng, Mercado-Uribe et al. 2010, Mullany, Fan et al. 2011, Mullany and Richards 2012). Nonetheless, some recent genetically modified models have arisen (Li, Mohanraj et al. 1992, Hahn, Stewart et al. 1999, Liu, Yang et al. 2004, Yang, Rosen et al. 2007, George, Kim et al. 2017). These models are valuable tools to study the aetiology and progression of ovarian cancer in a well-defined genetic background, but they do not incorporate all the physiological and synergic aspects of the normal human ovarian tissue microenvironment, including the hormonal control. Ovarian cancer models have been reviewed elsewhere (Rosen, Yang et al. 2009, Mullany and Richards 2012).

Cancer cell lines have been used for decades. They provide a rational model to develop therapeutic approaches for histological and molecular subtypes of cancer (Beaufort, Helmijr et al. 2014). A cell line, NIH: OVCAR-3, was established from malignant ascites of a patient with progressive ovarian adenocarcinoma and used by investigators since the 80s (Hamilton, Young et al. 1983). Genomic, proteomic and

biomarker features of cancer cell lines for ovarian carcinoma subtypes have been well established and investigated (Anglesio, Wiegand et al. 2013, Beaufort, Helmijr et al. 2014). OVCAR-3 cell line remains a good representative for epithelial high-grade ovarian serous adenocarcinoma. Moreover, these cultured cells contain cytoplasmic androgen- and estrogen-binding macromolecules with specificity for the respective steroid receptors hence maintaining the hormonal microenvironment typical of the ovary (Hamilton, Young et al. 1983). Thus, OVCAR-3 cells appear to be appropriate to study the nerve ovarian cancer connection *in vitro*, however the appropriate neuronal cell line for co cultures has not been developed.

The culture of neuronal cells is very challenging since adult neurons cell cycle is stagnant thus they do not undergo cell division (Gordon, Amini et al. 2013). Nonetheless, the recent availability of well-characterized and immortalized neuronal cell lines lead to a significant expansion of knowledge in neurobiology (Murayama, Singh et al. 2001, Gordon, Amini et al. 2013). Recently, Chen and co-workers immortalized and characterize a nociceptive dorsal root ganglion sensory neuronal line that promise to be useful for high-throughput drug screening for neuroprotective agents for axonal degeneration and anti-nociceptive drugs (Chen, Mi et al. 2007). This cell line membrane channels and hormonal modulators were further described (Vetter and Lewis 2010, Bhattacherjee, Liao et al. 2014). Plus, promising to be useful to study complex mechanisms regulating axonal outgrowth and integrity, under trophic factors stimulus and/or through cancer microenvironment neurochemical cues (Bhattacherjee, Liao et al. 2014, Pundavela, Demont et al. 2014).

Here, using OVCAR-3 model ovarian cancer cell line, I investigated the effects of different neurotransmitters in their morphology, invasion and migration properties, and their influence in neurite outgrowth in immortalized dorsal root ganglia cells 50B11. I

suggest that trophic factors other than NGF modulate the cancer cell-neuron interactions in ovarian cancer. Moreover, neurotransmitters present in cancer cell media were shown to affect cancer cell morphology.

6.2. Materials And Methods

Protocols were as outlined in chapter 2. Briefly:

6.2.1. Preparation Transwell invasion / migration assays

Cell invasion and migration assays were performed in 24-well Boyden micro-chambers (Transwell® Permeable Supports) with 8µm pore diameter membranes (#3422, Corning Inc., Cambridge, MA, USA), as previously described (Pundavela, Demont et al. 2014, Pundavela, Roselli et al. 2015, Katt, Placone et al. 2016).

After 62h incubation at 37°C with 5% CO_2, the Transwell inserts were processed for migration / invasion cell number counting.

Ovarian cancer cells invasion or migration properties were tested with NA 10µM, Ach 10µM, SP 1µM or 10µM formulation diluted in serum-free media.

6.2.2. Co-culture of immortalized dorsal root ganglia cells 50B11 and ovarian carcinoma cell line OVCAR-3

6.2.2.1. Neurite outgrowth assay

OVCAR-3 ovarian cancer cells were co-cultured with immortalized dorsal root ganglia cells 50B11. The 50B11 cells (50 000 cells/ml) were seeded on bottom wells

of 12-well Transwell™ plates (#3402 Corning Inc., Cambridge, MA, USA) coated with rat-tail collagen-I (Invitrogen, Australia). Ovarian cancer cells OVCAR-3 were grown in Transwell™ inserts (12.0 µm in diameter with 3 µm pores, Corning Inc.). Differentiation of 50B11 cells was allowed for 3 days or until neurites develop.

6.2.2.2. Morphology changes of cancer cells grown with neurotransmitters supplementation

Simplified morphological assays were performed with OVCAR-3 cells on 2D for split starved cells. Cell suspensions of 50 000 cells *per* ml of serum-free RPMI-1640 medium with added neurotransmitters NA, ACh or SP, in a range of concentrations (0.1, 1, 10, 50 and/or 100µM) were added to the wells of 12-well plates, and allowed to adhere and grow for 48-72h at 37°C and 5% CO_2 prior to fixation and imaging.

6.2.3. Statistical analysis

One-way ANOVA and Krustal-Wallis test were performed with cell counting data. Results were considered statistically significant with $p<0.05$. Statistical tests were carried out using GraphPad Prism version 6.0 (GraphPad Software, La Jolla California USA).

6.3. Results

6.3.1. Neurotransmitters vary in their ability to stimulate ovarian cancer cell migration in vitro

Neurotransmitters NA, ACh and SP were used to study their influence in ovarian cancer cell invasion and migration. OVCAR-3 cancer cells did not show invasion capability. I.e. OVCAR-3 cells do not secrete enzymes able to digest the collagen layer. However, they migrated normally through the Transwell system (Figure 6.1 A).

A shows representative images of ovarian cancer cells cultured in conditioned media with neurotransmitters. Starvation media and media with 10% FCS were also used as controls. The whole Transwell membrane inserts were counted due to low numbers of migrated cancer cells. SP did not stimulate cancer cell migration at either 1 or 10 µM concentrations. NA had limited effects in cell migration and thus was not distinguished from starvation conditions. Whilst, a substantial stimulation of ovarian cancer cells migration was observed in incubations with Ach 10µm (Figure 6.1 B).

6.3.2. Ovarian cancer cells induce morphologic changes in immortalized sensory neurons

I next investigated through co-cultures the cross-talk between ovarian cancer cells and immortalized sensory neuronal cells 50B11 (Figure 6.2). Elongation of 50B11 cells neurites was stimulated with forskolin, as previously described (Chen, Mi et al. 2007, Pundavela, Demont et al. 2014). OVCAR-3 cells induced strong neurite outgrowth (Figure 6.2) similar to that observed with 50µM forskolin. To test if NGF was responsible for the neurite outgrowth, a blocking antibody against NGF was used

(Figure 6.2). proNGF/NGF recombinant proteins also stimulated neurite outgrowth in 50B11 cells, as expected (Figure 6.2). OVCAR-3 cells induced neurite outgrowth similar to forskolin and proNGF/NGF recombinant protein (Figure 6.2). To test if OVCAR-3 cells induced neurite outgrowth by releasing NGF, a blocking antibody was used. In the presence of this blocking antibody combination, OVCAR-3 cells were still able to induce neurite outgrowth. Furthermore, proNGF/NGF recombinant protein and proNGF/NGF blocker increased the proliferation of 50B11 cells, and mainly cells of angular shape were observed (Figure 6.2 E)

6.3.3. Ovarian cancer cells morphology and division are dose-dependently affected by neurotransmitters in vitro

Ovarian cancer cells OVCAR-3 were cultured in starvation media with a range of concentrations of NA, ACh and SP, and fixed after 48h and 72h. Figure 6.3-6.5 show representative phase images of resultant ovarian cancer cells. Serum-starved cells and a control with FCS were also included. OVCAR-3 cells exhibit normal epithelial-like morphology assuming polygonal shape with regular dimensions that can be observed in small patches. Compared with FCS, starved cells were generally larger in size and in nuclear-cytoplasmic ratio, but barely multiplied after seeding (Figure 6.3-6.5). OVCAR-3 cells exhibited highly variable shapes including round, oval, elongated, and clusters, in the presence of all three neurotransmitters. Non-round and multi-nucleate cells were sometimes observed. OVCAR-3 cells showed more division and cells were smaller when cultured in media with NA 10 μM. Higher concentrations of NA reduced the number of cells suggesting cell division was inhibited (Figure 6.3 A & B), although cells appeared larger at 72 vs. 48h. (Figure 6.3) OVCAR-3 cells cultured in media with 10 μM Ach were larger in size and more numerous compared with starvation media

(Figure 6.4). Ovarian cancer cells in the presence of ACh showed also more oval and angular shapes. In 50µM ACh cells were, smaller, more polygonal, and less aggregated than those in 10 µM ACh (Figure 6.4). In the presence of SP, there were fewer OVCAR-3 cells compared with either NA or ACH incubation and cell size was smaller (Figure 6.5 A & B). Number of oval and angular cancer cells and luminous cells under phase-microscopy and in a representative area of the well observed at 20x objective is registered in Figure 6.6.

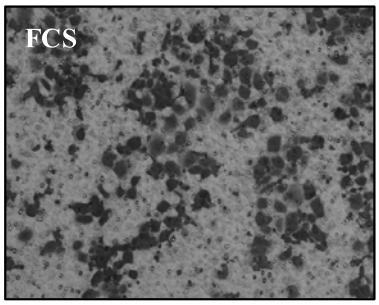
6.4. Discussion

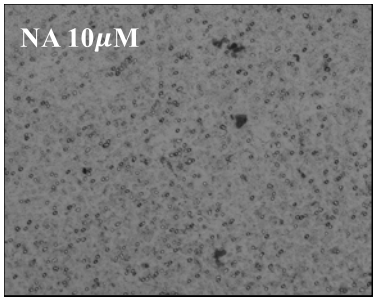
Cancer progression includes numerous steps. To become metastatic, a tumour must invade locally to then metastasise to distant sites. Local cancer cell invasion occurs first through the thin layer of basement membrane composed mainly of collagen IV and laminins, and then through a dense extracellular matrix mainly made of fibrillary collagen I (Friedl and Wolf 2003, Clark and Vignjevic 2015, Ziperstein, Guzman et al. 2015). Unlike our laboratory's published results for breast (Pundavela, Roselli et al. 2015) and prostate cancer cell lines (Pundavela, Demont et al. 2014) I found OVCAR-3 ovarian cancer cell line to be non-invasive. However, OVCAR-3 cells migrated in culture. I tested the effect of neurotransmitters NA, ACh and SP at various time points after seeding the OVCAR-3 cancer cells. NA had no notable effect in cancer cell migration and SP if anything inhibited it. ACh showed some evidence of increased migration through Transwell membranes especially at later time points, and the longer in culture the more this effect was observed.

I used the 50B11 neuronal cell line of sensory nociceptive cells to test the cross-talk between ovarian cancer cells and neurons. I found OVCAR-3 cells were able to stimulate 50B11 neurons neurite outgrowth. However, this was not driven by proNGF or NGF suggesting that other growth factors must be driving ovarian cancer cell stimulation of neuronal cells, e.g. GDNF family of trophic factors – CHAPTER 5: Our laboratory has previously used 50B11.cells in co-culture with prostate cancer cells to show that proNGF released from cancer cells promotes neurite outgrowth (Pundavela, Demont et al. 2014). One important caveat to my results is that 50B11 cells represent just one class of sensory neurons. And do not necessarily reflect the situation for autonomic neurons. Our laboratory has used PC12 cells in co culture with breast cancer cell lines. But these are not derived from a neuronal population. Finally, my observations from chapter 4 imply that a cholinergic neuron derived cell line (see (Rabinovsky, Le et al. 1992, McLaughlin, Tsirimonaki et al. 2006)), or a primary culture would be more appropriate to test the ability of ovarian cancers to attract axons.

Some workers have suggested that morphological changes including the nucleus to cytoplasmic ratio and cell shape can be a prognostic marker of invasiveness (Chen, Zhao et al. 2012, Park, Ang et al. 2014). For example, in breast cancer cell lines Kenny and colleagues (Kenny, Lee et al. 2007) suggested that aggregate size and nuclear cytoplasmic ratio could be used. My observations indicate that OVCAR3 cells in the presence of ACh form larger aggregates and maintain increased cell size compared with serum starved. Whereas in the presence of SP cells were smaller and did not divide. Further analysis of the effects of neurotransmitters and growth factors on cell morphology may be warranted.

Figure 6.1 – Effects of neurotransmitters on cell migration of OVCAR-3 cells. (A) Representative images of OVCAR-3 cells in Transwell® membranes. In the presence of foetal calf serum (FCS) there was marked migration of OVCAR-3 cells through the transwell membrane, as judged by the presencve of cells in the underside of membrane (Image A, top left). In serum-free media (Image A, bottom left) very few OVCAR-3 cells migrated to the underside of the membrane. In the presence of 1 or 10µM SP or 10µM NA migration was similar to that observed in serum-free conditions. More cells migrated in the presence of 10µM Ach (Image A, bottom right) but this migration did not approach that observed in FCS conditions. (B) Quantification of migration number in four replicates. Scale bar = 100 µm.


FCS


NA 10μM

B.

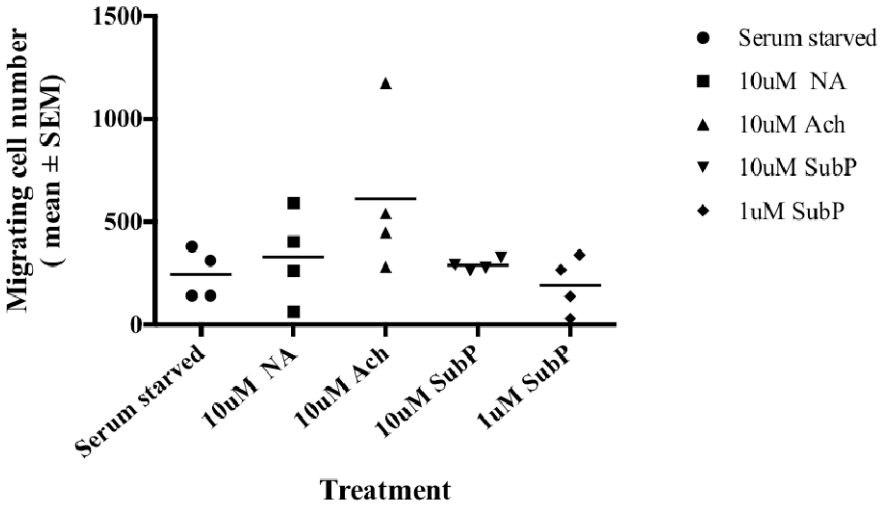
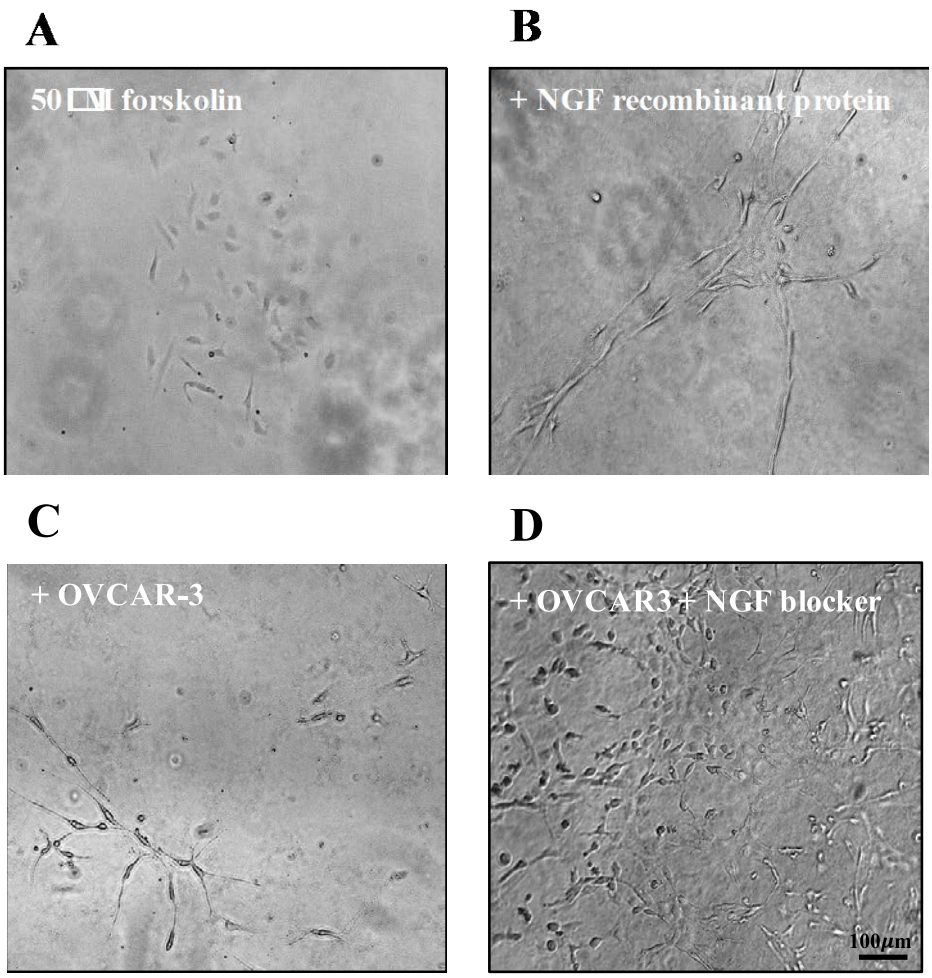

Migrating cell number
(mean ± SEM)
1500
1000
500
0
Serum starved
10uM NA
10uM Ach
10uM SubP
1uM SubP
Treatment
Serum starved
10uM NA
10uM Ach
10uM SubP
1uM SubP

Figure 6.2 – Neurite outgrowth of 50B11 immortalized sensory neurons. (A) 50µM forskolin induced neurite outgrowth. (B) NGF recombinant protein also induced neurite outgrowth. (C) The presence of OVCAR-3 cells stimulated neurite outgrowth similar to NGF. (D) Incubation with NGF blocking antibodies did not inhibit OVCAR-3 induced neurite outgrowth. Scale bar = 100 µm. (E) Contingency bar graph showing number of oval cells and angular cells in the field of view/image. When in the presence of forskolin 50B11 cells differentiate and extend neurites. 50B11 cells also differentiate in the presence of proNGF/NGF and of OVCAR-3 cancer cells. Interestingly,, in the presence of OVCAR-3 cells, NGF blockers did not prevent the sprout of neurites by neuronal cells and higher proportion of angular cells was also observed.


A
50 μM forskolin
B
+ NGF recombinant protein
C
+ OVCAR-3
D
+ OVCAR3 + NGF blocker
100μm

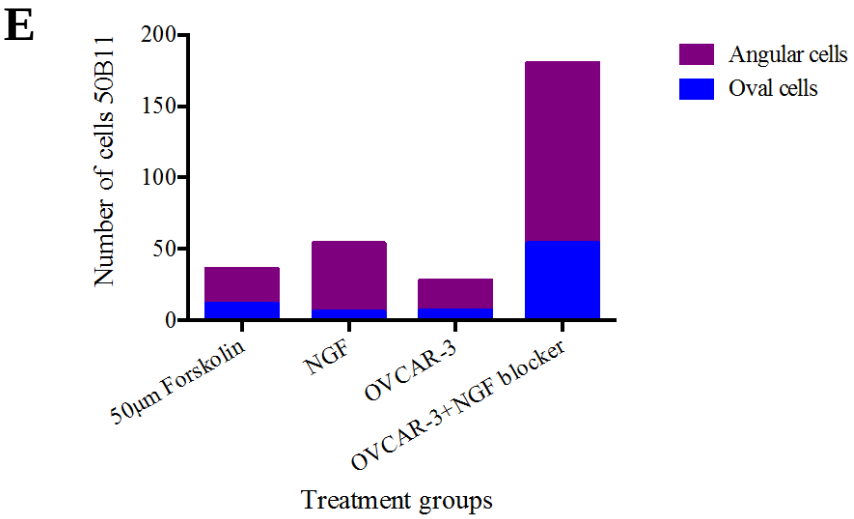
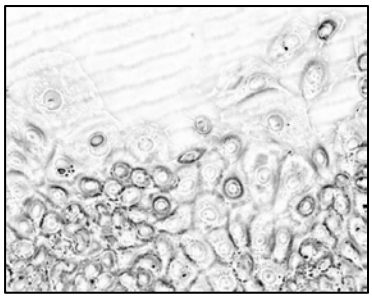
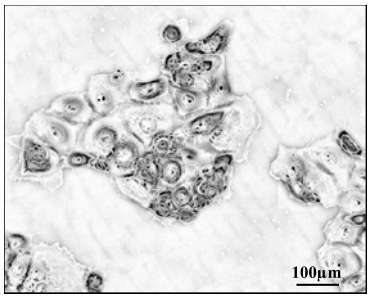
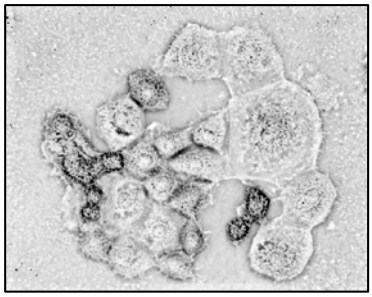

E
Number of cells 50B11
200
150
100
50
0
50μm Forskolin
NGF
OVCAR-3
OVCAR-3+NGF blocker
Treatment groups
Angular cells
Oval cells

Figure 6.3 – Representative images of the ovarian cancer cells OVCAR-3 in the presence of different concentrations of neurotransmitter NA. (A) After 48h in 0.1μM NA I observed large aggregates of small cells with regular round to oval shape. Increasing concentrations of NA resulted in a smaller cell size and reduced number of cells indicative of increased cell death or inhibited cell division. (B) After 72h in NA, the cell morphology and number was similar at NA concentrations 0.1-10μM (Image B, top right). At higher concentrations of NA (50μM) cells reduced in number and abnormal morphology. In serum starved media, cells were of similar morphology but fewer in numbers compared with NA treatments. Scale bar = 100 µm.




100µm



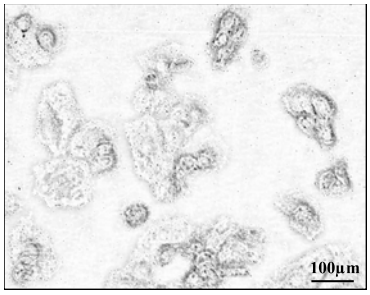
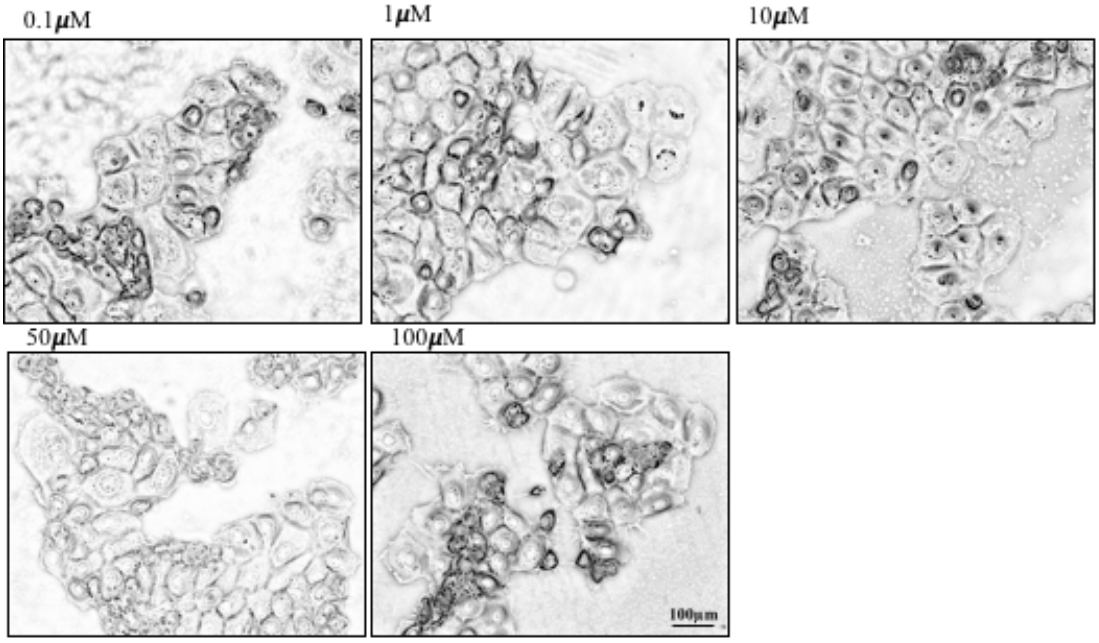

100µm

Figure 6.4 - Representative images of the ovarian cancer cells OVCAR-3 in the presence of different concentrations of neurotransmitter Ach. (A) After 48h in 0.1μM Ach I observed large aggregates of cells with polygonal to round shape. Increasing concentrations of Ach resulted in reduced number of cells and smaller shape. (B) After 72h in Ach, the cells showed larger size, identical morphology and decreased number (Image B). At higher concentrations of Ach (50μM) cells showed smaller size and abnormal morphology. In serum starved media, cells were of similar morphology but fewer in numbers compared with Ach treatments. Scale bar = 100µm.

A. Incubation with Acetylcholine during 48h



B. Incubation with Acetylcholine during 72h

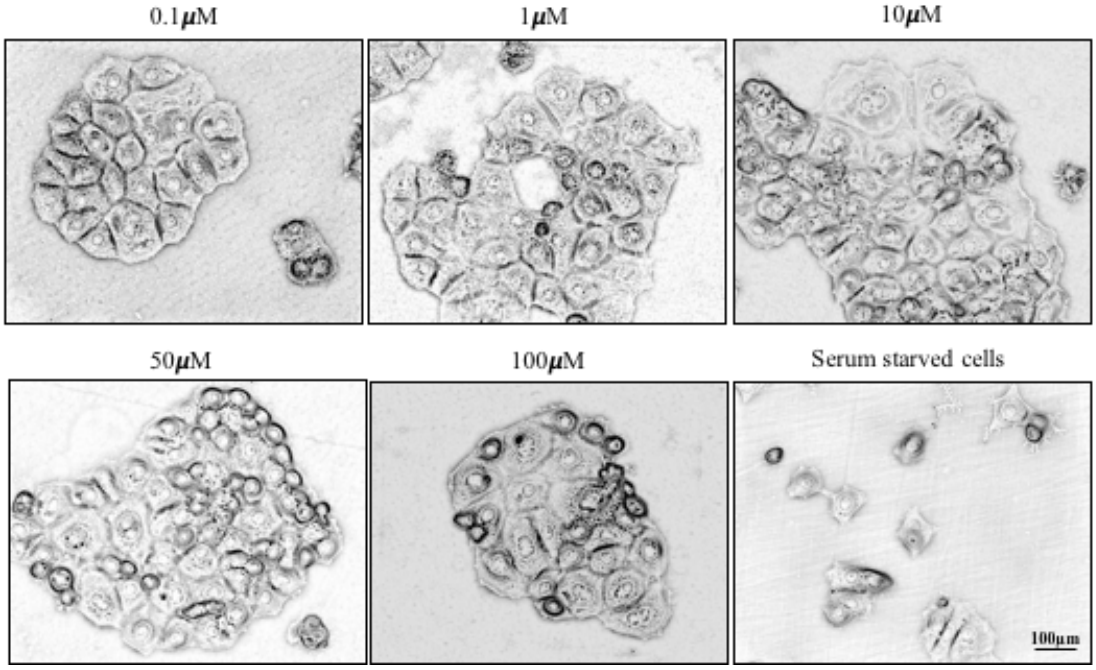
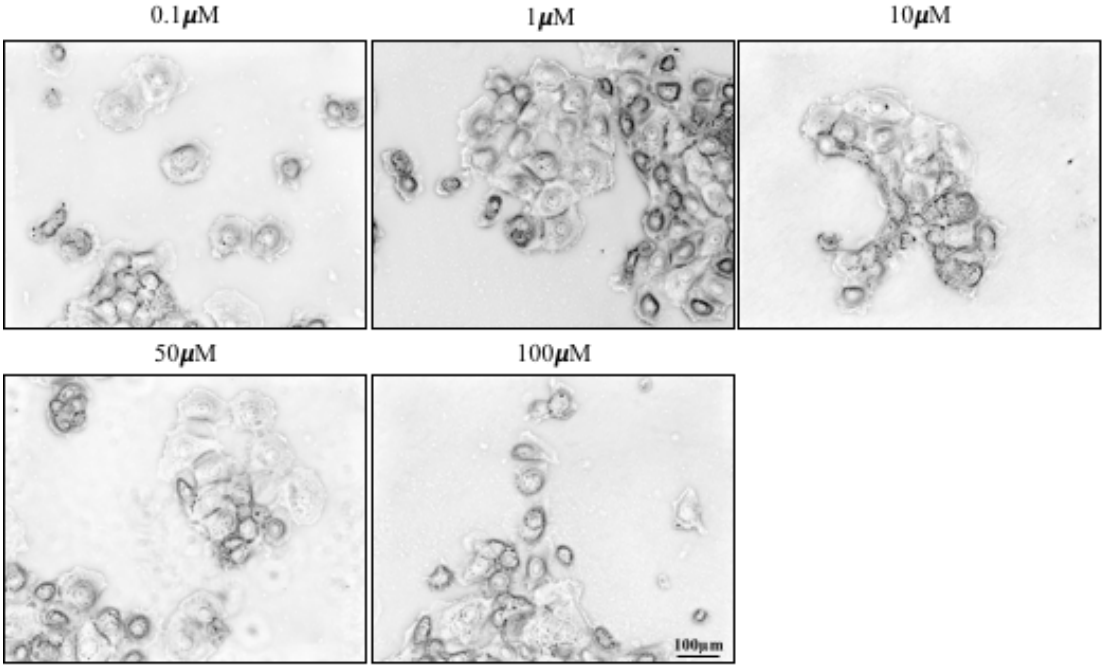


Figure 6.5 - Representative images of the ovarian cancer cells OVCAR-3 in the presence of different concentrations of neuropeptide SP. (A) After 48h in 0.1μM SP I observed individual and small aggregates of cells with regular to round shape. Increasing concentrations of SP resulted in reduced number of cells and smaller size. (B) After 72h in SP, the cells showed larger size, identical morphology and decreased number (Image B). At higher concentrations of SP (50μM) cells showed smaller size and abnormal morphology. In serum starved media, cells were of similar morphology but fewer in numbers compared with SP treatments. Scale bar = 100µm.

A. Incubation with Substance P during 48h



B. Incubation with Substance P during 72h

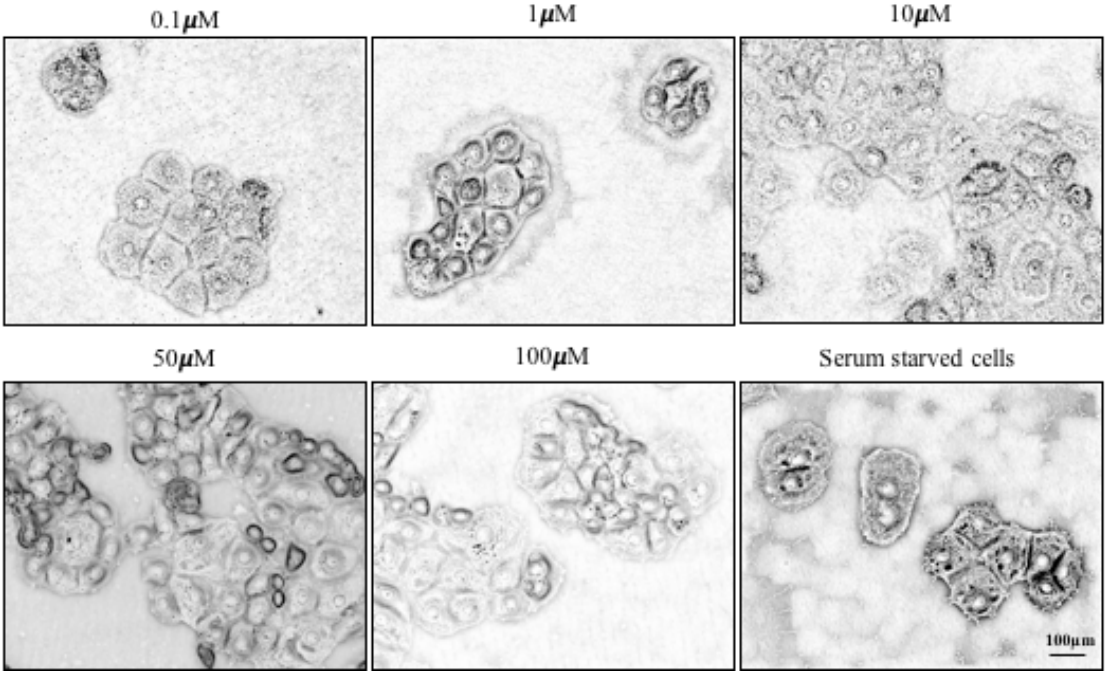
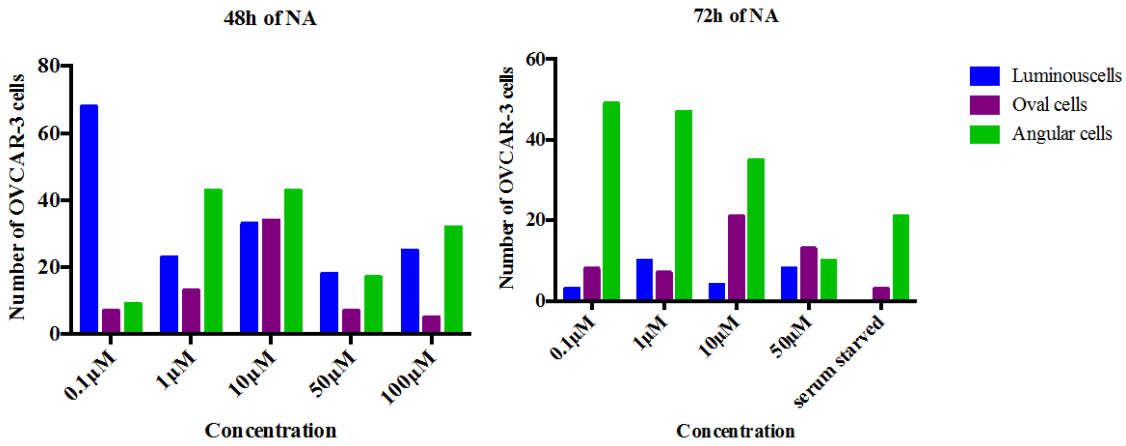
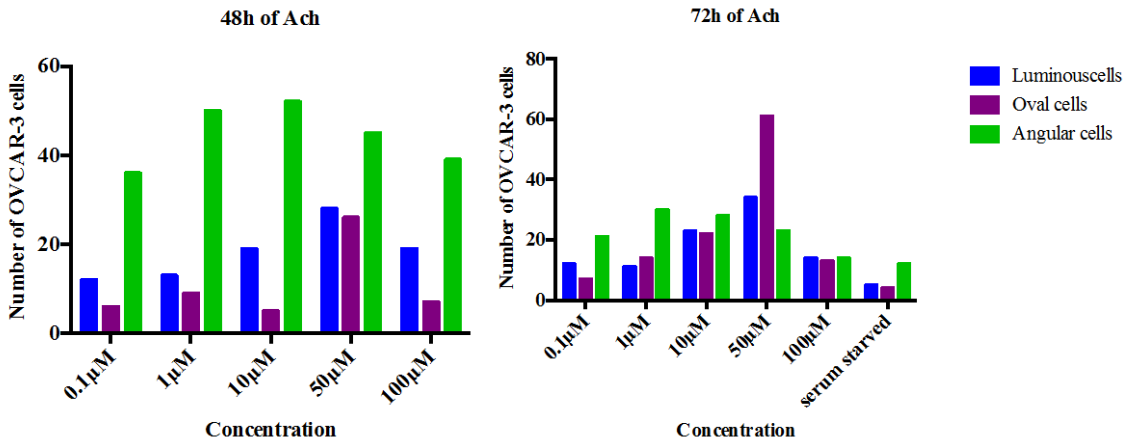


Figure 6.6 - Cell morphology analysis from phase-contrast optical images of OVCAR-3 cells cultured with neurotransmitters in a gradient of concentrations and duringat two time-points: 48h and 72h of incubation. Cells showing high luminiescence and cells of angular or oval shape were counted. (A) Concentrations of NA over 10µM resulted in reduced number of cancer cells. At 72h of incubation, cell survival and/or proliferation present recovery. (B) Concentrations of Ach over 50 µM are detrimental for OVCAR-3 cells, which presented optimal growth with 10µM of Ach in serum-free culture media. (C) Unlike NA or Ach, SP presence in serum-free culture media promoted cell survival and proliferation best at 1µM of SP at 48h, but of 10 and 50µM SP at 72h of incubation.

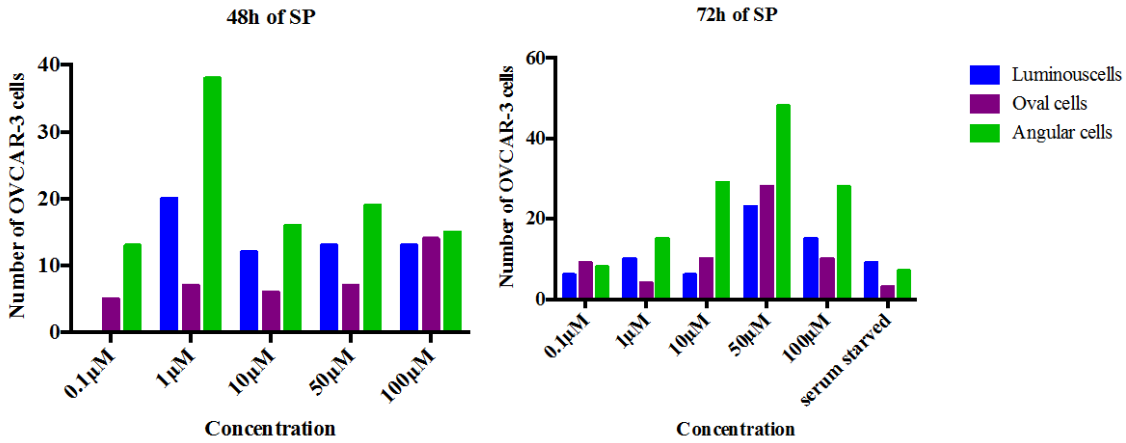
A. Incubation with noradrenaline



B. Incubation with acetylcholine



C. Incubation with substance P



CHAPTER 7: CERVICAL TUMOURS ARE INNERVATED AND EXPRESS NEURONAL MARKER(S)

7.1. Background

In Chapters 3 and 4, I demonstrated that peripheral nerves were not a feature of the ovarian tumour microenvironment compared with other solid tumours, like breast and prostate tumours. Whether this is due to the unique properties of the nerves that innervate the pelvic viscera in that they do not respond to growth factors released from tumours or the unique profile of growth factors released from cancer cells is unknown. Therefore, in this chapter I investigated another gynaecological cancer.

The aetiology of cancer of the cervix differs somewhat from breast and ovarian cancer with a strong association with HPV infection (Burd 2003, Schiffman, Castle et al. 2007, Angiolo, Silvestro et al. 2017). In the context of the current study, the cervix is heavily innervated by sensory axons that play a major role in parturition (Puder and Papka 2005). Intriguingly, the interplay between local inflammation, recruitment of immune cells and parturition is intrinsic to normal cervix function. Whether invasion of cervix tumours by sensory (or autonomic) axons impacts disease progression is unknown but forms the rationale for this chapter.

Anatomically, the cervix is essentially made of fibrous connective tissue composed mostly of extracellular matrix (ECM) dominated by collagen and proteoglycans

(Ferenczy 1982, Granstrom, Ekman et al. 1989). During pregnancy and labour, and even sex, the innervation to the cervix and local neurotransmitter content has been described to rearrange and change density (Houdeau, Rousseau et al. 1998, Collins, Usip et al. 2002, Mowa and Papka 2004). Given that axons projecting to the cervix under normal conditions are able to grow and retract depending on reproductive state, it would be interesting to see if tumours of the cervix attract a higher innervation density compared with ovarian tumours.

7.2. Materials And Methods

All protocols are identical to those described for PGP9.5, NGF and proNGF labelling in chapter 2 and chapter 3.

7.2.1. Cervix tissue samples

Tumour microarrays (TMAs) of cervical cancers and adjacent normal and/or normal cervical tissue (CR6161) were obtained from US Biomax, Inc. (Maryland, USA). This cohort englobed 294 cases of cervical carcinoma with duplicate cores *per* case and 28 adjacent normal (NAT, normal adjacent to tumour) and normal tissue in a single tissue core *per* case. The cohort investigated included 257 squamous cell carcinoma cases and 30 adenocarcinomas. Plus 4 rare cervical adenosquamous carcinomas, 2 clear cell carcinomas and one undifferentiated carcinoma. For PGP9.5, core biopsies analysed consisted of 27 adjacent normal and normal tissue and 294 cervical carcinomas. For NGF and proNGF. Core biopsies analysed consisted of 28 NAT cores and 287 carcinoma samples.

7.3. Results

7.3.1. The cervical tumour microenvironment includes peripheral nerves

Here, I used the pan-neuronal marker to look for axons and neuronal cells in a cohort of cervical tumours. Histologically cores of normal cervix revealed consistent innervation (82% of samples; Table 7.1) across the fibromuscular tissue and near vasculature (Figure 7.1). The cervical cancer histology was substantially different from that of observed in normal cervix biopsies (Figure 7.1. Likely our samples came from the transformation zone, area between original squamocolumnar (SC) junction and new SC junction due to regenerative metaplastic response; site of > 90% of squamous cell carcinomas and dysplasia (Herfs, Vargas et al. 2013, Akbar, Tunio et al. 2016). Axons were observed near vasculature and around cancer cell groups, including in bundles (Figure 7.1). PGP9.5-IR was also expressed in neuroendocrine cells and cancer-associated fibroblasts (Figure 7.1 and Figure 7.2 C). PGP9.5-IR was also observed in cancer cells. Few tumours were PGP.5 –IR negative (7% of tumours; Table 7.1). All adenosquamous tumours were PGP9.5-IR positive, while 97% of adenocarcinomas and 92% of squamous cell carcinomas exhibited PGP9.5-IR (Table 7.1) therefore, not pinpointing a difference across the different types of cervical tumours. Higher presence of innervation was recorded in younger patients (p=0.0386; Table 7.1). However, no other significant correlation was detected between clinicopathological parameters and PGP9.5-IR. (Table 7.1).

7.3.2. Expression of neurotrophins in cervix cancer

I next determined the expression of NGF and proNGF in normal and cancerous cervix biopsies. NGF was expressed at low levels in normal cervix with mean h-score of 45.91 ± 3.61, n=28 (Table 7.2, Figure 7.3 B). NGF expression was significantly higher in cervix tumours (with an h-score of 80.14 ± 8 (n=30) in adenocarcinoma and 98.47 ± 2.17 (n=116) in squamous cell carcinoma (Table 7.2). In terms of clinicopathological features there was an association with tumour grade where higher grade tumours had increased NGF expression (Table 7.2).

The intensity of proNGF –IR was also significantly increased in cancerous cervix tissue with an h-score of 147.00 ± 1.34, n=287 compared with 116.50 ± 6.37, n=28 in normal tissue ($p < 0.0001$, Table 7.3, Figure 7.4 B). Mean h-score was slightly, yet significantly, higher in squamous cell carcinoma (148.10 ± 1.38, n=257) than adenocarcinoma (137.70 ± 4.63, n=30, $p=0.0165$) – Table 7.3, Figure 7.4 B. Similar to NGF expression, higher grade tumours had increased proNGF mean h-score (Table 7.3).

7.4. Discussion

The majority, 87% of all cervical tumours studied had axons marked by the pan neuronal marker PGP9.5. The nature of these axons warrants further investigation. I predict that these axons will be derived from both sensory and autonomic neurons. As expected, the majority (75%) of normal cervix biopsies also had axons. The fact that not all patients had observable axons may point to the relative size of the core biopsy

compared with axon density. Three of these patients' biopsies revealed PGP9.5-IR neuroendocrine cells only.

The presence of nerves was not statistically linked to disease progression, nor to cancer pathology. However, a higher proportion of younger patients showed innervation of cervical tumours. Innervation of the reproductive tract is influenced by hormones and is known to change with reproductive status (Monica Brauer and Smith 2015).

The expression of both NGF and proNGF was increased in cervix cancer. As there is a high expression of nerves in normal and cancerous tissue there is no obvious association between these neurotrophins and axon invasion. In addition, while NGF expression may be required during development, the low levels of NGF in normal cervix suggests that maintenance of innervation does not require high levels of NGF.

Finally, while there was no association between axon invasion and cancer or axons and neurotrophin expression, the association between NGF and proNGF and cancer was significant. This suggests that anti-neurotrophin treatment might be a future therapeutic option for these patients.

Table 7. 1 - Association between PGP9.5-immunoreactivity and clinicopathological parameters of cervical tumours. For each category, the number of cases is indicated and the corresponding percentage is under brackets. Statistically significant p-values ($p<0.05$ using chi-square test) are shown in bold and were confirmed in Log linear analysis for normal vs cancer and histological types.

PARAMETER	PGP9.5 NEGATIVE	PGP9.5 POSITIVE	P-VALUE
All cases (n=321)			
Normal (n=27)	4 (14%)	23 (82%)	0.1814
Cancer (n=294)	22 (7%)	272 (93%)	
Pathological subtype			
Squamous cell carcinoma (n=257)	21 (8%)	236 (92%)	0.5403
Adenosquamous (n=4)	0	4 (100%)	
Adenocarcinoma (n=30)	1 (3%)	29 (97%)	
Clinical parameters of tumours			
Patient Age			
≤ 50 (n=181)	9 (5%)	172 (95%)	**0.0384**
> 50 (n=113)	13 (12%)	100 (88%)	
Tumour size (T)			
Squamous cell carcinoma			
T<3 (n=251)	21 (8%)	230 (92%)	0.4597
T>3 (n=6)	0	6 (100%)	
Adenosquamous			
T<3 (n=4)	0	4 (100%)	-
T>3 (n=0)	-	-	
Adenocarcinoma			
T<3 (n=30)	1 (3%)	29 (97%)	-
T>3 (n=0)	-	-	
Tumour grade			
Low (grade 1; n=31)	2 (6%)	29 (94%)	0.5047
High (grades 2-3; n=228)	19 (8%)	209 (92%)	
Undetermined (n=35)	1 (3%)	34 (97%)	
Lymph Node Invasion (N)			
Negative (n=273)	20 (7%)	253 (93%)	0.8721
Positive (n=20)	2 (10%)	18 (90%)	
Undetermined (x) (n=1)	-	1 (100%)	
Nerves			
Negative (n=37)	20 (54%)	17 (46%)	**NA**
Positive (n=257)	0	257 (100%)	

Table 7. 1 - IHC h-scores for NGF-immunoreactivity in a series of cervical tumours versus normal tissues TMAs, and associations with clinicopathological parameters . NGF immunohistochemical staining in each sample was digitally quantified and h-score (mean ± standard error of the mean) is reported. Statistically significant p-values (p<0.05, using t-tests and ordinary one-way ANOVA) are shown in bold. h-score varied with histological subtype and with tumour grade (p<0.05). Unlike what was observed in ovarian cancer (Figure 3.4, page 96), low grade serous tumours registered lower median h-score. Cervical tumours also show significant higher h-score than normal cervix.

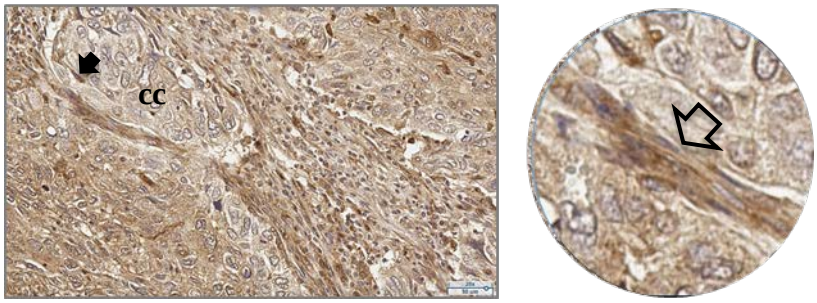
PARAMETER	NGF INTENSITY (H-SCORE) MEAN	STD. ERROR	*P*-VALUE
Normal vs. Cancer			
Normal (n=28)	45.91	3.61	**< 0.0001**
Cancer (n=287)	96.56	2.13	
Cancer Histological Subtype			
Adenocarcinoma (n=30)	80.14	8.00	**0.0083**
Squamous (n=257)	98.47	2.17	
Age (Years)			
<50 (n=171)	99.71	2.70	0.0726
≥50 (n=116)	91.91	3.45	
Tumour Size (T)			
T1 + T2 (n=281)	96.34	2.16	
T3 + T4 (n=4)	110.60	15.33	0.4344
Missing (n=2)	98.52	37.54	
Lymph Node Status (N)			
Negative (n=266)	96.80	2.22	
Positive (n=20)	95.10	8.31	0.8399
Missing (n=1)	60.98	0.00	
Stage			
I + II (n=257)	96.73	2.26	0.7960
III + IV (n=28)	94.86	6.85	
Missing (n=2)	98.52	37.54	
Grade			
1 (n=29)	79.59	6.32	
2 (n=168)	98.81	2.69	**0.0017**
3 (n=62)	108.30	4.67	
Missing (n=28)	74.54	5.63	

Table 7.2 - IHC h-scores for proNGF-IR in a series of cervical tumours versus normal tissues TMAs, and associations with clinicopathological parameters . proNGF immunohistochemical staining in each sample was digitally quantified and h-score (mean ± standard error of the mean) is reported. Statistically significant p-values (p<0.05, using t-tests and ordinary one-way ANOVA) are shown in bold. h-score varied with histological subtype and with tumour grade (p<0.05).

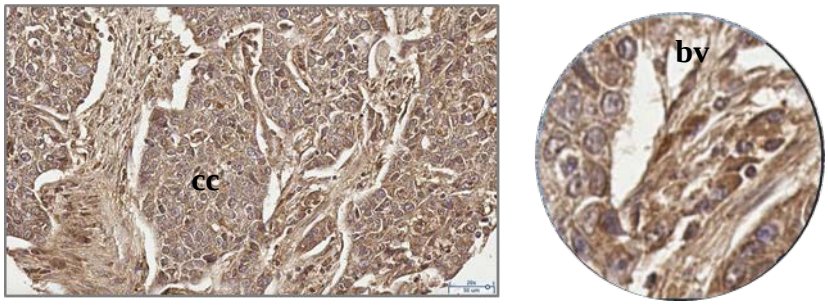
	PRONGF INTENSITY (H-SCORE)		
PARAMETER	**MEAN**	**STD. ERROR**	***P*-VALUE**
Normal vs. Cancer			
Normal (n=28)	116.50	6.37	**< 0.0001**
Cancer (n=287)	147.00	1.34	
Cancer Histological Subtype			
Adenocarcinoma (n=30)	137.70	4.63	**0.0165**
Squamous (n=257)	148.10	1.38	
Age (Years)			
<50 (n=171)	149.10	1.66	0.0679
≥50 (n=116)	144.10	2.22	
Tumour Size (T)			
T1 + T2 (n=281)	147.20	1.36	
T3 + T4 (n=4)	138.80	6.69	0.4606
Missing (n=2)	135.60	1.82	
Lymph Node Status (N)			
Negative (n=266)	147.30	1.42	
Positive (n=20)	143.80	3.99	0.5092
Missing (n=1)	137.40	0.00	
Stage			
I + II (n=257)	147.70	1.44	
III + IV (n=28)	141.90	3.56	0.2054
Missing (n=2)	135.60	1.82	
Grade			
1 (n=29)	132.70	4.14	
2 (n=168)	151.20	1.60	**< 0.0001**
3 (n=62)	150.20	2.61	
Missing (n=28)	130.00	4.89	

Figure 7.1 – Detection of axons in human cervical cancer using the pan-neuronal marker PGP9.5. (A) Squamous cervical tumour showing PGP9.5-IR in cancer cells (cc), axons (arrow) and cancer-associated fibroblasts (black arrow). (B) Adenosquamous cervical tumour showing widespread PGP9.5-IR in cancer cells. (C) Adenocarcinoma cervical tumour also showing PGP9.5-IR in cancer cells, cancer-associated fibroblasts and nerve bundles (arrow). (D) In normal cervix, PGP9.5-IR was more often restricted to small axon profiles (arrow) and seldom in endothelial cells '(white arrow). Images on the right are magnifications of the ones showed in the left panel. Scale bar = 50 µm.

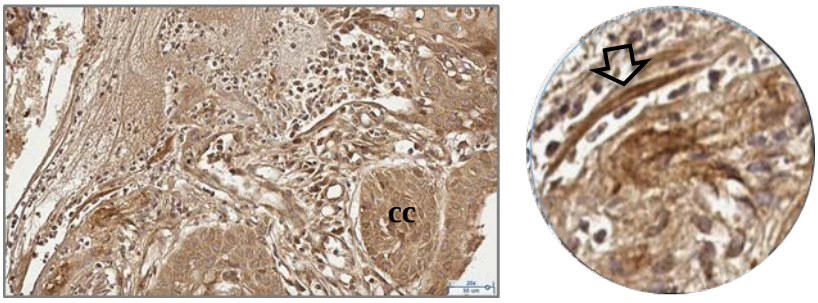
A. Squamous CeCa



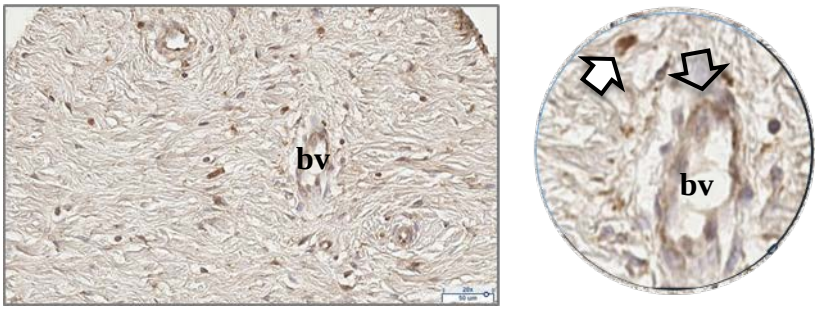
B. AdenoSquamous CeCa



C. AdenoCarcinoma



D. Normal cervix



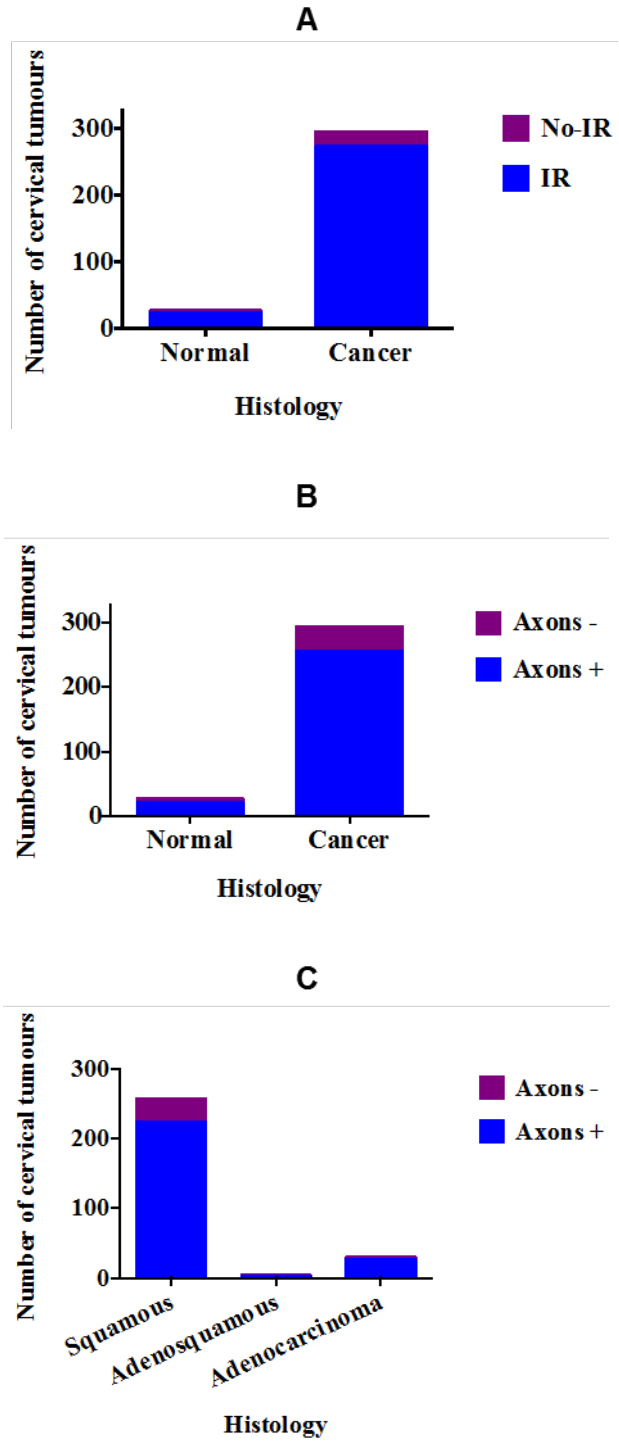
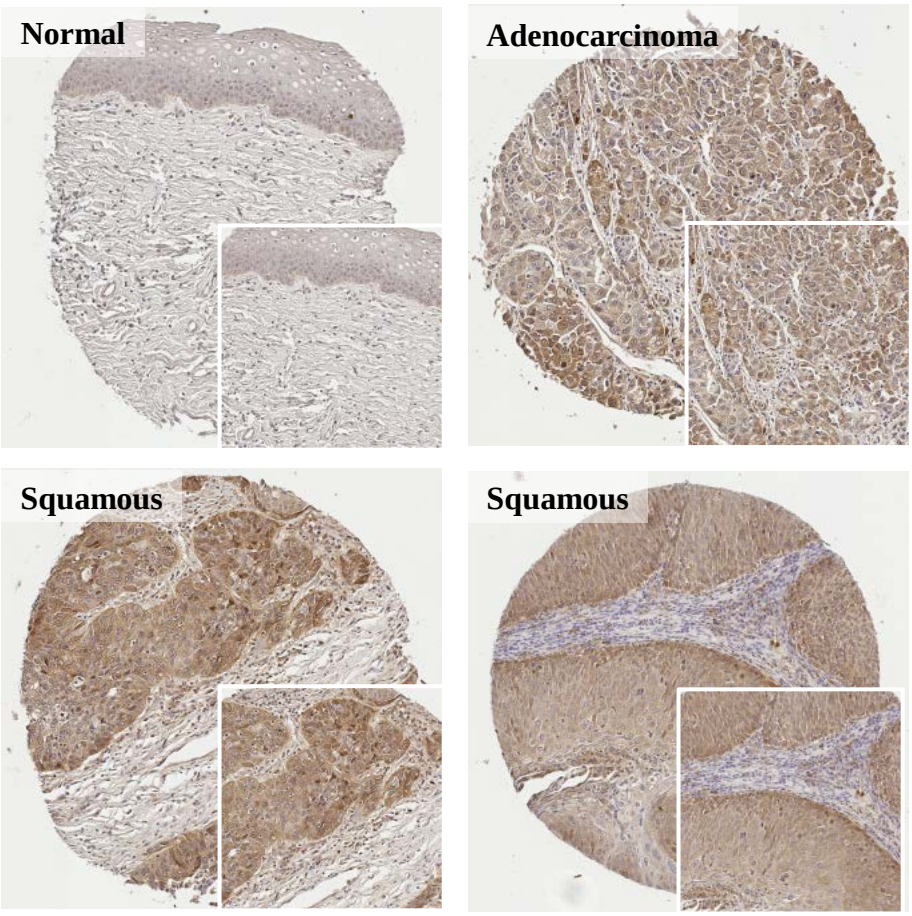


Figure 7. 2 – Quantification of the pan-neuronal marker PGP9.5 in human cervical tissue-microarrays. (A) PGP9.5-IR across any cell type in normal cervix tissue and in cervical tumours. (B) Number of cases showing PGP9.5-IR axons in cervical tumours. (C) Relative presence of axons according to tumour type. Axons were prevalent in all tumour types.

Figure 7.3 – Nerve growth factor (NGF) in the cervix tumour microenvironment. (A) Cervix tumours showed variable NGF-IR but normal cervix showed little NGF-IR Images scanned at 10x and zoomed area in the corners. (B) IHC h-scores for NGF-IR in a series of cervix tumours TMAs. (A) h-score median was significantly higher in cervix tumours (h-score showed as mean ± standard error of the mean, and p<0.0001 between normal cervix and tumours, using t-tests and ordinary one-way ANOVA).

A.


Normal
Adenocarcinoma
Squamous
Squamous

B.

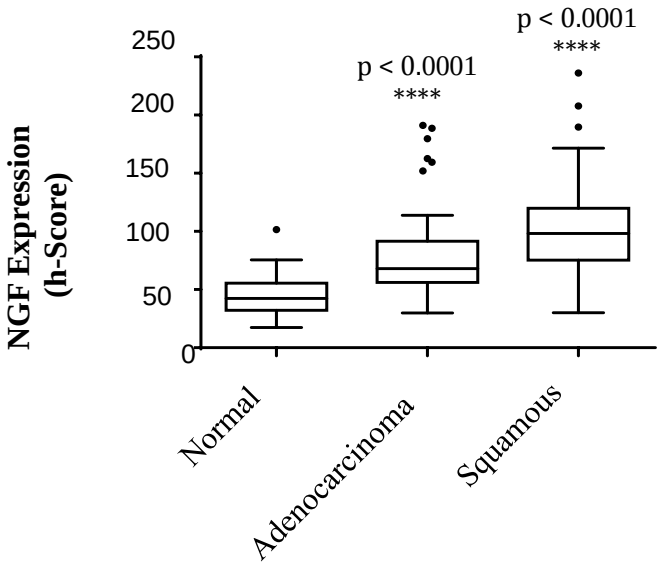
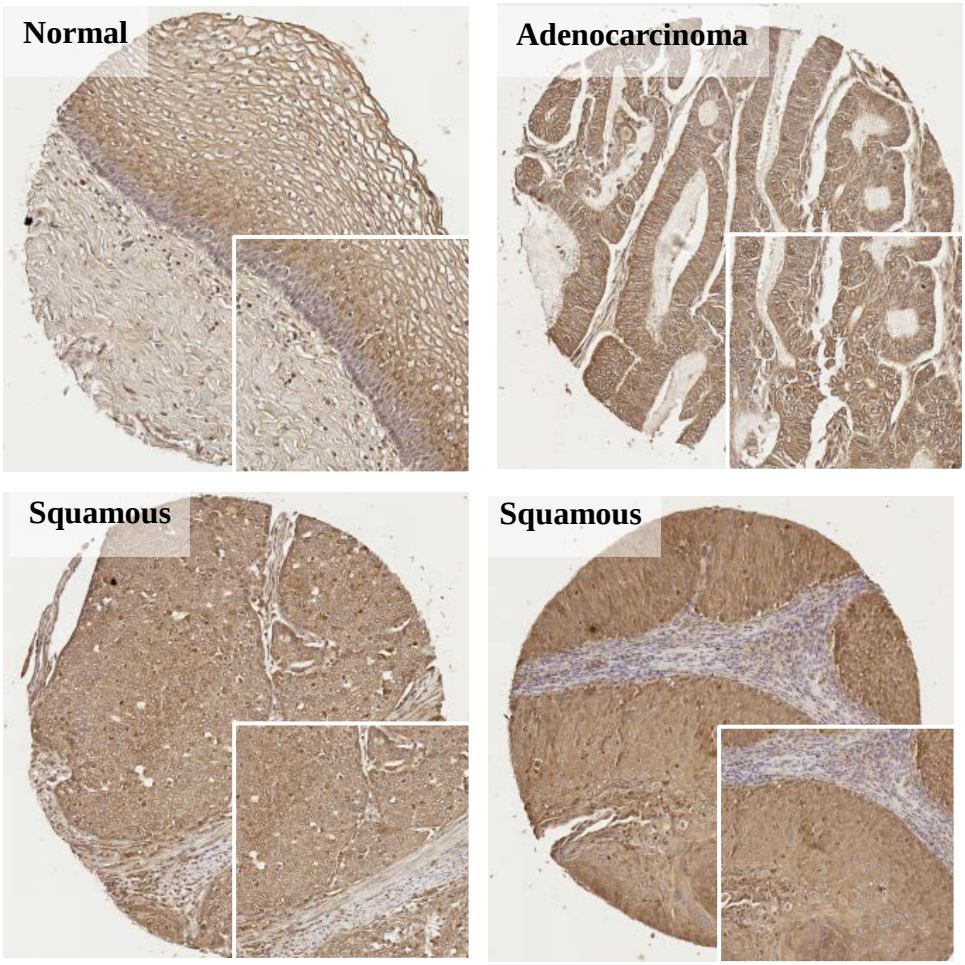
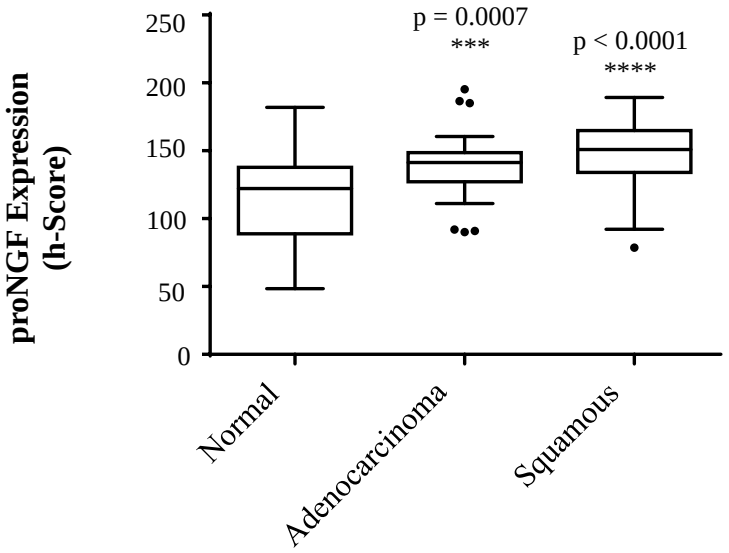

NGF Expression
(h-Score)
250
200
150
100
50
0
p < 0.0001
****
p < 0.0001
****
Normal
Adenocarcinoma
Squamous

Figure 7.4 – Precursor nerve growth factor (proNGF) in the cervix tumour microenvironment. (A) Cervix tumours showed low proNGF-IR variability. Images scanned at 10x and zoomed area in the corners. (B) IHC h-scores for proNGF-IR in a series of cervix tumours TMAs. (A) h-score median was significantly higher in cervix tumours (h-score showed as mean ± standard error of the mean, and significant p values between normal cervix and tumours determined using t-tests and ordinary one-way ANOVA).

A.


Normal
Adenocarcinoma
Squamous
Squamous

B.


proNGF Expression
(h-Score)
250
200
150
100
50
0
p = 0.0007
***
p < 0.0001
****
Normal
Adenocarcinoma
Squamous

CHAPTER 8: GENERAL DISCUSSION

8.1. Nerves and cancer

The nerve cancer connection is a relatively new paradigm that is becoming established based on clinical observations and supported by *in vivo* and *in vitro* experimental models. Evidence is strongest for prostate and breast cancer where several laboratories have found evidence for axonal sprouting into tumours. In addition, gastric cancer and pancreatic cancer are thought to respond to substances released from nerves. Data mainly from our laboratory suggest that neurotrophins may attract axons from adjacent normal tissue into the tumour microenvironment. The overall aim of my project was to see if this emerging principle extended to ovarian cancer.

One of the challenges of this study is that unlike the male reproductive tract or uterus, cervix and vagina in females, innervation of the normal ovary remains an enigma. Ovarian autonomic innervation, as with innervation of all pelvic structures, is complex arising from an extensive plexus distributed along the female reproductive tract and associated organs and within the ovary. As this plexus receives input from both lumbar and sacral spinal cord segments the distinction between sympathetic and parasympathetic pathways becomes blurred (Keast 1999, Keast 2006). Nevertheless, I predicted that as with prostate cancer axons arising from the hypogastric plexus, which are thought to form part of the normal innervation, nerves would sprout into ovarian tumours. Moreover, I predicted that this might be associate with more aggressive cancers.

A summary of the growth factors or receptors that were present in the ovarian tumour microenvironment is shown in Figure 8.1.

While I certainly found peripheral axons in core biopsies from many ovarian tumours, sympathetic axons as determined by TH-IR were relatively rare. When innervation was considered as a whole, and assuming that PGP9.5 is able to detect most peripheral nerves, I again saw less innervation than reported for breast and prostate tumours, and no obvious association with clinicopathological features. It should be pointed out that I examined an equivalent number of tumour core biopsies as reported in studies on breast cancer and prostate cancer where clear associations were found. Therefore, it seems that the nerve-cancer connection in ovarian cancer is not equivalent to other solid tumours. One major caveat to the above conclusion is that the cell types in ovarian cancer and the difference in cancer cell to stroma ratio in varies between mucinous and serous cancer and is markedly different to breast or prostate tumours. Investigating whole human ovarian tumours for the presence of axons has considerable logistical and ethical constraints. But seeing if axons along the periphery of tumours are correlated with clinical outcome would be informative. *In vitro* exposure to NA resulted in minimal stimulation of ovarian cancer cells migration although some morphological changes in these cancer cells, namely size and perceived division ratio suggesting OVCAR -3 cells express adrenoceptors.

The identity of the non-adrenergic axons observed in ovarian tumours was also determined. I used an antibody raised against vAchT. vAChT is widely accepted as an appropriate marker for peripheral cholinergic axons (Arvidsson, Riedl et al. 1997). It is arguably a better gauge of sites of ACh release from axons compared with choline acetyl transferase. However, while I was able to find axons with vAChT-IR labelling,

it was also observed in cancer cells and small neuroendocrine-like cells. Non-neuronal cholinergic signalling is now widely accepted within the immune system. Likewise, also in normal ovarian function (Stefenson, Owman et al. 1981, D'Albora, Lombide et al. 2000, Anesetti, Lombide et al. 2001, Kozlowska, Majewski et al. 2014). However, whether this is necessarily through a normal vesicular release has not been determined. Use of other markers such as CHAT combined with markers of muscarinic and nicotinic receptors expression will need to be employed to further probe the pathophysiological significance of Ach in tumour progression.

Promising is the observation that ACh can effectively stimulate ovarian cancer cell migration. Moreover, ovarian cancer cells in the presence of ACh showed a strong pre-disposition to multiply even at high concentrations.

The focus of previous studies on the nerve-cancer connection has been on the autonomic nervous system (Magnon, Hall et al. 2013, Zhao, Hayakawa et al. 2014, Zhao, Yang et al. 2014, Pundavela, Roselli et al. 2015). However, peripheral sensory neurons also respond to neurotrophins and neurotransmitters released from sensory nerve endings and have the capacity to affect the tumour microenvironment (Laird, Roza et al. 2001, Palecek, Paleckova et al. 2003, Pintado, Pinto et al. 2003, Erin, Boyer et al. 2004, Erin, Zhao et al. 2006, Lindsay, Halvorson et al. 2006, Jimenez-Andrade, Bloom et al. 2010). While a number of sub-populations of sensory neurons innervate pelvic organs, peptidergic neurons containing SP and CGRP have been observed in all mammals studied. I predicted that SP-IR axons may be part of the ovarian tumour nerve population. However, while SP-IR was sparsely observed in the ovary it was generally rare and not confined to axons. SP also forms part of a non-neuronal signalling system in some tissues (Lang, Drell et al. 2003, Katsanos, Anogeianaki et al. 2008, Rittner,

Brack et al. 2008). Interestingly, the preferred receptor for SP the NK1 receptor was expressed in many cancer cells. Although these receptors might not encounter substantial amounts of SP, the possibility of other neurokinins (NKA, NKB) activating the receptor cannot be excluded. Also, the molecular subtype of these NK1 receptors need to be determined (Jobling, Messenger et al. 2001, Baker, Morris et al. 2003).

A link between SP and cancer migration/progression has been suggested (Seckl, Higgins et al. 1997, Munoz and Covenas 2013). Furthermore, the SP-NK1 pathway has been associated to underlie intrinsic proliferative and inflammatory signalling pathways in other cancers and disease (Candenas, Lecci et al. 2005, da Silveira, Freitas et al. 2008, Thomson, Terman et al. 2008, Clement, Peeters et al. 2009, Rudick, Schaeffer et al. 2009, Gozal, Kim et al. 2014). Therefore, the fact that I observed very few SP-IR axons in ovarian cancer suggest that the lack or disruption of this sensory signalling pathway may encompass a reason for the lack of specific pain symptoms associated with ovarian cancer diagnosis.

Plus, SP showed no significant effect on ovarian cancer migration. As stated above complete characterization of neurokinin receptor subtypes will need to be performed to pursue any therapeutics targets based on tachykinins.

Other neurotransmitters and synaptic co-factors will also need to be investigated. An example is neuronal NOS. Unfortunately, while our nNOS antibody was a selective marker for neurons and axons in our reference tissue human gut, expression of nNOS in cancers was widespread and difficult to interpret. So, the role of nNOS axons whether autonomic or sensory remains to be determined. Whether cancer cells were expressing genuine nNOS is not known, nor are the implications of this finding for cancer progression and treatment.

8.2. Neurotrophins and cancer

Neuronal growth factors are important for follicular assembly, maturation and ovulation, and dysregulation of these have been associated with abnormal ovarian morphology (Sirotkin 2011, Streiter, Fisch et al. 2015). That neurotrophins may promote cancer progression independent of nerves was established several decades ago (Descamps, Toillon et al. 2001, Montano and Djamgoz 2004, Vanhecke, Adriaenssens et al. 2011, Hondermarck 2012). Recent work from our laboratory confirms the link between NGF and/or proNGF and cancer progression. It also suggests that part of this, but potentially not all, is due to acceleration of nerve invasion. I found NGF to be expressed by cancer cells in a number of ovarian tumours. However, this was not associated with clinical outcome, nor was NGF evenly distributed amongst tumour grades. In addition, NGF was also not associated with more tumour innervation. A similar pattern emerges with proNGF, which again sets ovarian tumours apart from breast or prostate cancer. NGF/proNGF can stimulate immortalized DRG sensory cells to grow neurites. Similar was observed when these cells were co-cultured with ovarian cancer cells. However, differently from breast or prostate cancer cells, blocking antibodies against NGF/proNGF epitopes did not prevent neuronal cells from sprouting neurites in the presence of ovarian cancer cells. Thus, suggesting other neurotrophic factors are at play.

In order to probe the potential role of neurotrophins and other nerve growth factors in cancer progression I looked for the expression of a range of receptors.

8.2.1. Neurotrophic factors receptors and ovarian cancer

Both mature and pro-neurotrophins use dual-receptor complexes. Mature neurotrophins such as NGF engage with the respective Trk receptor, TrkA and $p75^{NTR}$, wherein Trk transduces signals for survival and differentiation, and $p75^{NTR}$ regulates the affinity and selectivity of Trk activation. Pro-neurotrophins engage $p75^{NTR}$ and the co-receptor sortilin to initiate $p75^{NTR}$-dependent signal transduction cascade that may result in cell death (Teng, Felice et al. 2010). Hence, $p75^{NTR}$ activation is pivotal for deciding apoptotic and non-apoptotic responses.

However, I could not find a correlation between NGF and TrkA expressions in ovarian cancer. Sortilin-IR in the normal ovary was nil and non-significant in ovarian cancer. All the ovarian tumours studied were $P75^{NTR}$-IR positive hence, suggesting that ovarian cancer cells specificity and/or selection to specific neurotrophins or grown factors is determined by tyrosine kinase receptors being expressed.

Ovarian tumours showed high expression of TrkB and of receptors for the GDNF family (GFRs), which includes GDNF, NRTN and ARTN as ligands. Thus, neurotrophins other than NGF may be major contributors to the mechanisms of ovarian cancer.

Similarities and potential overlapping of GDNF and NGF families pathways were suggested before to regulate the development of the peripheral nervous system (Baloh, Enomoto et al. 2000). Possibly, identical overlapping pathways may exist in the ovarian tumour microenvironment.

The presence of GFRs in the GCs of human primordial follicles was previously reported (Farhi, Ao et al. 2010). However, Farhi and colleagues only investigated the expression of GFRα1 and RET. This is the first work to report the protein expression of the receptors for neurturin and artemin, GFRα2 and GFRα3 respectively, and my

results suggest that the GDNF family could have a significant role in the ovarian tumour microenvironment.

Together, our data suggest that GDNF may be involved in the regulation of primordial follicular activation and to play a role in ovarian cancer progression and ovarian innervation rearrangement. Namely, through the GDNF-RET complex and GFRα1 released by nerves and/or expressed by cancer cells as suggested by (He, Chen et al. 2014) using pancreatic cancer cells.

GDNF, as well as NGF, are produced by cardiac and smooth muscle cells and have important roles in the sensory and autonomic innervation of blood vessels. Neural innervation is essential for blood vessel function (as discussed in Chapter 1). Plus, the same cues are involved in the embryonic patterning of the developing vertebrate vascular and peripheral nervous systems (Carmeliet 2003, Weinstein 2005).

(Zhong, Gu et al. 2016) reported GDNF overexpression in hepatic cancer cells, a non-neuronal tissue resistant to anti-angiogenic treatment using the VEGF antibody. I observed GDNF receptor GFRα1 overexpressed in specific ovarian cancer cells. Thus, it is possible that GDNF may be an important factor promoting pathological neovascularization and neoneurogenesis, in simultaneous. If this applies, GDNF may be a potential target in ovarian cancer and existent therapies targeting both neovascularization and neoneurogenesis may offer preferred therapeutical options.

8.3. Comparison with another gynaecological cancer

As outlined above the nerve-cancer/neurotrophins connection is not as strong in ovarian cancer compared with breast, prostate, gastric or pancreatic cancers. This could conceivably be either a function of the particular axons surrounding the tumour or a function of the ovarian microenvironment. Therefore, I chose another well-known target of paracervical nerves, the cervix, for comparison. PGP9.5 protein expression was analysed by IHC. PGP9.5-IR was extensively observed in both cervical tumours (over 90% of tumours) and in the normal cervix (~80%). No statistical difference was detected between normal and cancer of the cervix.

These results indicate that some axons innervating pelvic viscera readily invade tumours. Moreover, nerves are a feature of the cervical tumour microenvironment. Despite the presence of axons in most of the cervical tumours I could not find an association between either cancer stage or lymph node involvement. However, the small number of patients that had lymph node involvement means that our study is underpowered, and I cannot exclude a role for nerves in cervical cancer progression.

Some key questions still remain regarding axon infiltration in cervical cancer. Obviously, the origin of axons, whether they are autonomic or sensory, and importantly whether they are derived from lumbar or sacral spinal outflows need to be determined.

While there was no association between axon invasion and cancer, or axons and proNGF and NGF expression, the association between these neurotrophins and cervical cancer was significant.

To deepen the question, there are studies that show that proNGF and NGF effects could be regulated by balance expression of their receptors, as indicated by in vitro studies in PC12 cells (rat pheochromocytoma) (Ioannou and Fahnestock 2017). I.e. proNGF is neurotrophic under normal circumstances but loss of TrkA with P75NTR

and Sortilin receptors expression unchanged can drive cell death (Ioannou and Fahnestock 2017). In samples of cervical cancer patients, both protein expression of NGF and proNGF follow the same trend. Their immunoreactivity is significantly higher than in normal cervix. The mechanism requires further investigation, but neurotrophins could offer potential therapeutic tools to gynaecological cancer patients.

Hormonal status can also be important as it has been showed to regulate NGF in a manner that varies among tissues (Bjorling, Beckman et al. 2002). Consequently, hormonal fluctuations along with expression of NGF and proNGF and other factors may impact inflammatory response, tumour outcome and pain sensation (Bjorling, Beckman et al. 2002, Hassan, Muere et al. 2014). Thus, further characterization of the tumour microenvironments of ovarian and cervix cancers is complex but necessary.

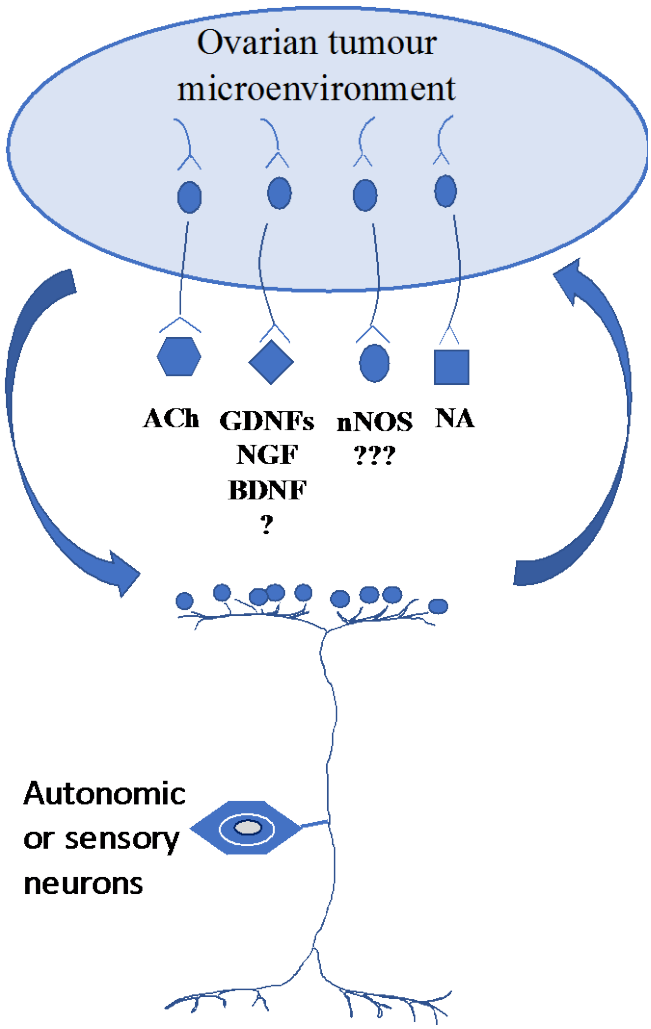


Figure 8.1 – Schematics of the nerve-cancer crosstalk in ovarian cancer. Ovarian cancer cells express receptors for neurotransmitters as SP (SP binds to NK1 receptors where it activates de PKC signalling pathway), and for neurotrophic factors including NGF (TrkA receptor), BDNF (TrkB receptor), and GDNF family (GFR receptors α1, 2 &3). Nerves in the tumour microenvironment release neuronal factors that activate receptors expressed by the cancer cells. If this offers a selective advantage, cancer cells that establish communiction with the axons will be selected and the tumour progresses.

8.4. Conclusion

This thesis has provided the first description of peripheral nerves in the ovarian cancer microenvironment. Three populations of neurons were found both in biopsies of normal and tumours of the ovary. vAChT-IR axons showed significant presence while TH-IR axons were seldom observed. It is likely that non-cholinergic and non-adrenergic axons, probably sensory were present. However, few SP-IR axons were observed despite the presence of NK1. TrkA and TrkB, $p75^{NTR}$ and sortilin growth factors receptors were also present although with no clear correlation to clinicopathological parameters TrkB expression, deserves better exploration. GFRα1,2,3 receptors were described for the first time in ovarian tumours and their role should be investigated due to their relevant expression in ovarian cancer cells. The above data suggest that ovarian tumours are likely to be different from breast, prostate, gastric and pancreatic tumours with respect to nerves and neurotrophins. Supporting the unique nature of ovarian tumours was the finding that cervix cancers had more axon infiltration than ovarian tumours. Neurotrophin expression in cervical cancer also follows different trend from ovarian cancer. This further suggests that the ovarian cancer microenvironment is relatively unique compared with other solid tumours, including other gynaecological cancers.

CHAPTER 9: FUTURE PERSPECTIVES

Ovarian cancer in women is a complex and deadly disease whose research is further impaired by difficulties in sample/patient control and the lack of physiologically meaningful models. Adding to scantily understood nerve-hormone interaction, molecular events that also initiate and control tumour formation and progression remain poorly defined. Mouse models probably remain the better option to study mechanisms by which specific oncogenic factors or genes cause the different types of ovarian cell modifications (Thaker, Han et al. 2006, Mullany and Richards 2012, Gao, Foster et al. 2015).

The current mouse models provide a reasonably good platform to study transformation in the ovarian stem cell populations, regarding molecular or ontogenetic traits. Furthermore, they exhibit similar histology to the various ovarian cancer subtypes found in women. However, mouse models of high-grade ovarian carcinomas are still lacking (Mullany and Richards 2012). So far, the role of axons or neurotrophins in any ovarian cancer animal model is unknown.

The mechanisms by which cancer cells and neurons communicate are probably best studied in vitro where experiments can be highly controlled, and changes in neuronal properties easily quantified. While I observed stimulation of neurite outgrowth by OVCAR-3 cancer cells similar to that promoted by breast and prostate cancer cell lines as reported by Pundavela and colleagues (Pundavela, Demont et al. 2014, Pundavela, Roselli et al. 2015) no one has yet looked to see if these morphological changes are associated with a change of neuronal excitability. Such experiments may provide insight into why some cancers are painful and others not.

Similarly, other features of the tumour microenvironment such as extracellular matrix molecules, including netrins and slits, could be studied in the context of neurons and neurotrophins. For example, the importance of NGF in the ovarian tumour innervation may be related to the co-expression of a particular extracellular-matrix molecule and not just its correspondent receptors (i.e. TrkA and $p75^{NTR}$). Such an approach should also be explored.

Thus far, most ovarian cancer cell lines show heterogeneity namely in the morphology, molecular characteristics (Beaufort, Helmijr et al. 2014) and response to growth factors stimulation (Bourgeois, Kabarowski et al. 2015). Moreover, different ovarian cancer cells lines ought to be tested, in particular a cell line of invasive cancer cells (e.g. SKOV3.ip1 (Bai, Feng et al. 2006)). This thesis presents the first body of work, to the best of my knowledge, exploring the nerve-cancer crosstalk in the ovarian cancer cell line OVCAR-3 which is non-invasive and slow-migrating. Thus, it is important to extend this work to other well-characterized cell lines.

In summary, there is a range of therapeutic targets that remain to be explored to achieve our goal of reducing the burden of gynaecological cancers.

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