# UNIVERSITAT de BARCELONA

# Combined Therapies for Neuroblastoma Based on the Activation of the Calcium-Sensing Receptor

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# Combined Therapies for Neuroblastoma Based on the Activation of the Calcium-Sensing Receptor

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## Summary

Neuroblastoma (NB) is the most common extracranial solid childhood tumour. Its clinical and histological manifestations range from benign tumours that spontaneously regress to highly aggressive metastatic tumours (*Matthay* et al - *2016*). In spite of the advances in treatment and the multidisciplinary approaches, almost half of high-risk NB patients do not survive (*Whittle* et al – *2017*). The Calcium-sensing receptor (CaSR) is a G-protein coupled receptor (GPCR) that was found to be expressed in good prognosis NB tumour (*de Torres* et al – *2009*). Activation of this receptor using cinacalcet (CIN), a positive allosteric modulator of CaSR, in a xenograft NB animal model, reduced tumour growth. In addition, it was described that in NB cell lines with an overexpression of CaSR, CIN induced ER-stress mediated apoptosis (*Rodríguez-Hernández* et al – *2016*). However, efficacy of CIN in the treatment of NB is limited by the low expression of CaSR in high-risk NB. Furthermore, CIN acts mainly in the CaSR present in the parathyroid glands, inducing hypocalcemia.

This work addresses these two limitations of using CIN in the treatment of NB patients. We demonstrate that the active compound of vitamin D, 1,25-dihydroxyde vitamin D (1,25-$D_3$) and the retinoids all-*trans*-retinoic acid (ATRA) and 13-*cis*-retinoic acid (13-*cis*-RA) increase the expression of *CaSR* in two NB cell lines. Unfortunately, *in vitro* anti-tumorigenic capacities of CIN are not increased by its combination with 1,25-$D_3$. Moreover, we show that the strong *in vitro* and *in vivo* anti-tumorigenic capacities of retinoids are unaltered by its combination with CIN.

On the other hand, we identify another positive allosteric modulator of CaSR, AC-265347, with NB anti-tumorigenic properties which does not induce hypocalcemia in mouse animal models. In these models each calcimimetic induce a differential protein and gene expression pattern, suggesting a different mechanism of action in the inhibition of tumour growth. Additionally, *in vitro* studies show that CIN and AC-265347 induce different stages of differentiation in NB cell lines.

Altogether, our data shows that the combination of CIN with different drugs that induce an increase in the expression levels of *CaSR* do not potentiate the anti-tumorigenic effect of CIN. More importantly, this work identifies a new calcimimetic that shows a potential neuroblastoma specific effect, AC-265347 inhibits NB tumour growth while maintaining plasma calcium levels. Our results strongly suggest that

AC-265347 may be an effective therapeutic agent against NB, alone or in combination with other potentially synergistic treatments.

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## List of Abbreviations

1,25-D$_3$ - 1,25-dihydroxycholecalciferol

13-*cis*-RA – 13-cis retinoic acid

7TM – Seven transmembrane domain

ADCY8 – Adenylate cyclase type 8

ATF4 – Activating transcription factor 4

ATRA – All-*trans* retinoic acid

BID – BH3 interacting domain death agonist

BSA – Bovine serum albumin

Ca$^{2+}$ – Calcium

Cl$^-$ – Chlorine

CaCl$_2$ – Calcium Chloride

cAMP – Cyclic adenosine monophosphate

CaSR – Calcium sensing receptor

CD44 – Cluster of differentiation 44

CHD5 – Chromodomain-helicase-DNA-binding protein 5

CHOP – C/EBP homologous protein

CIN – cinacalcet

CMD - C-terminal transmembrane domain

Col1A1 – Collagen type I

Col3 – Collagen type III

CSTA – Cysteine protease inhibitor

CTAs – Cancer testis agents

CTCFL – CCCTC-binding factor

CTGF – Connective tissue growth factor

DAG – Diacylglycerol

DMSO - Dimethyl sulfoxide

DMSO – Dimethyl sulfoxide

ECD – Extracellular domain

EFS – Event-free survival

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

EMT – Epithelial mesenchymal transition

ER – Endoplasmic reticulum

GAGE12J – G antigen 12J

GDP – Guanine diphosphate

GPCR – G-protein couple receptor

GRP78 – Binding immunoglobulin protein

GTP – Guanine triphosphate

H&E – Haematoxilin Eosin

HES1 – Hairy and enhancer of split-1

HSJD – Hospital Sant Joan de Dèu

ICD – Intracellular domain

ID2 – DNA-binding protein inhibitor

IHC – Immunohistochemistry

IL – Interleukine

IP3 – Ionositol 1,4,5-triphosphate

JNK – c-Jun N-terminal kinases

kb – Kilobase

kDa – Kilo Dalton

Log – Logarithm

MAGEA – Melanoma-associated antigen

MMP2 – Matrix metalloproteinase-2

MYCN – N-myc proto-oncogene

Nanog – Homeobox protein NANOOG

NB – Neuroblastoma

NEFL – Neurofilament light polypeptide

NGFR – Low-affinity nerve growth factor receptor

NOXA – Phorbol-12-myristate-13-acetate induced protein 1

NY-ESO-1 – Cancer-testis agent 1

Oct4 – Octamer-binding transcription factor 4

P1 – Promoter 1

PBS – Phosphate-buffered saline

PDX – Patient derived xenograft

PFA – Paraformaldehyde

PIP2 – Phosphatidylinositol 4,5-biphosphate

PKC – Protein kinase C

PLC – Phospholipase c

PRKCA – Protein kinase C alpha type

PTH – Parathyroid hormone

PUMA – p53 upregulated modulator of apoptosis

RT – Room temperature

Rt-qPCR – Real-time quantitative polymerase chain reaction

RYR – Ryanodine receptor 1

S100β –S100 calcium-binding protein B

SHPT – Secondary hyperparathyroidism

Snail – Zinc finger protein SNAI

Sox2 – SRY (sex determining region Y)-box 2

SSX4/4B – Synovial sarcoma breakpoint 4

STAT – Signal transduced and activator of transcription

TBP – TATA-box binding protein

TGFβ – Transforming growth factor beta

TGFβR – Transforming growth factor beta receptor

TNFα – Tumour necrosis factor α

Trib3 – Tribbles homolog 3

TrkA – Tropomyosin receptor kinase A

TrkC – Tropomyosin receptor kinase C

TUBB3 – Tubulin beta 3 gene

Tubβ3 – Tubulin beta 3 protein

UTR – Untranslated region

VDR – Vitamin D receptor

VDRE – Vitamin D receptor elements

VEGF – Vascular endothelial growth factor

VEH – Vehicle

VFT – Venus fly trap

VIM – Vimentin

# I. Introduction

# 1    Neuroblastoma

Neuroblastic tumours comprise of a heterogeneous group of developmental malignancies of the sympathetic nervous system that include neuroblastoma (NB), ganglioneuromas and ganglioneuroblastomas. NB is the most common extracranial solid tumour diagnosed in childhood (*Maris - 2010*) and in Europe it represents 10% solid tumours diagnosed in children under 15 years (Data from Orphanet Consortium). According to the Spanish Registry of Childhood Tumours (Registro Español de Tumores Infatiles – RETI-SEHOP) 2065 children were diagnose with NB from 1980-2017, making it 9.3% of all diagnosed childhood tumours (Informe Estadístico RETI-SEHOP, 1980-2017). Clinical evolution of NB ranges from spontaneous regression to a highly aggressive, chemo resistant disease. Long-term survival of malignant (high-risk) NB is less than 50% despite intensive multimodal treatment.

## 1.1   Origins of neuroblastoma

Historically, adult and paediatric cancers have been understood as the same disease. Technological advances allowed for better understanding of the molecular and biological features of adult and childhood cancer, showing different mechanisms of tumorigenesis. Paediatric cancers were shown to have a lower rate of somatic mutations than adult tumours, however epigenetic modifications and genomic instability are more common (*Lawrence* et al – *2013, Gröbner* et al – *2018*). Adult tumorigenesis process is the result of an accumulation of mutations that provide the cells tumorigenic capacities. This is a slow process that occurs along the lifetime of an individual and it is influenced by environmental and genetic factors (*Hanahan* et *Weinberg – 2011*). On the contrary, evidence from genetically engineered mouse models suggests that paediatric tumours originate from stem or progenitor cells during development time windows (*Funato* et al - *2014*). However, not enough is known about the tumorigenic drivers of these developmental failures (*Chen* et al – *2015).*

NB is thought to originate from the neural crest cells of the developing sympathetic nervous system. During embryogenesis neural crest cells are formed in the dorsal area of the neural tube and differentiate into several cell lineages. Cells undergo epithelial-mesenchymal transition (EMT), where they lose their polarity and

adhesion properties allowing them to delaminate, migrate and differentiate into a wide range of cell types, contributing to anatomical structures within the organism. Neural crest cells differentiate to different types of cells including components of the peripheral nervous system (sympathetic neurons, parasympathetic neurons, sensory neurons, enteric neurons, Schwann cells and satellite glia), medullary secretory cells, smooth muscle cells, melanocytes, bone and cartilage cells (*Sauka-Spengler* et *Bronner-Fraser – 2008; Matthay* et al *– 2016*) (Figure 1). These processes involve a complex modulation of cellular pathways and gene expression, including DNA methylation, histone modification and expression of bone morphogenic proteins
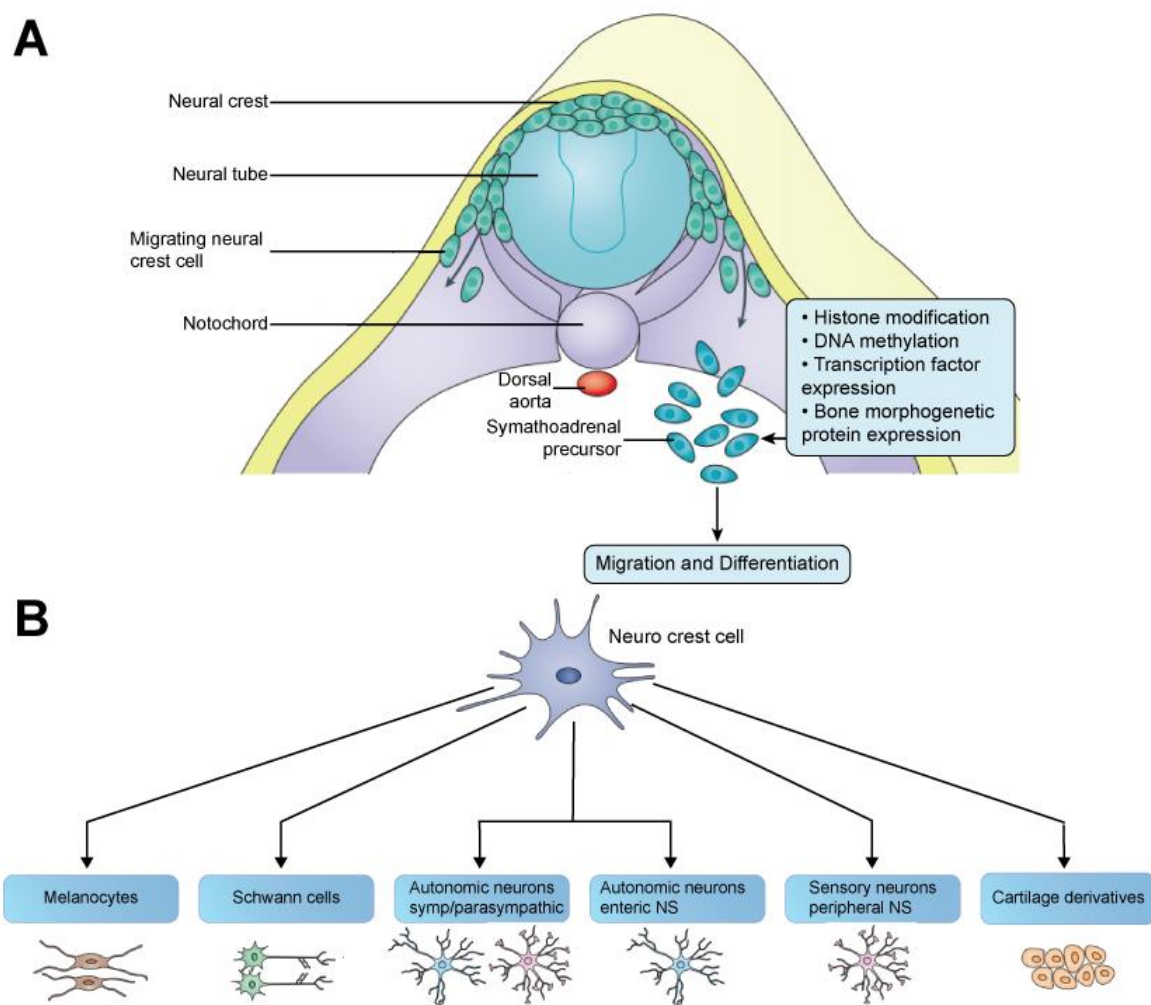


**Figure 1 – Neural crest terminal differentiation – A –** Neural crest is formed by the closing of the neural tube. Cells from this embryonic organ migrate to other areas of the embryo and differentiated into different cell type (adapted from *Matthay* et al *– 2016*). **B –** Differentiation of cells of neural crest different types of cells important in nervous system, skin and bone (adapted from *Sauka-Spengler* et *Bronner-Fraser – 2008*).

(BMPs) (*Kholodenko* et al *– 2018*). Dysregulation of factors involved in these

processes can induce changes in cell specification giving rise to hyperneoplastic lesions that eventually can result in neuroblastoma.

The most accepted hypothesis for the origin of NB is a failure in the differentiation of sympathoadrenal progenitors that will originate the sympathetic nervous system. Some of the evidences supporting this hypothesis are:

**1)** Location where tumours arise (47% in the adrenal gland or sympathetic ganglia, 24% in retroperitoneal area, 15% in thorax) (*Vo* et al – *2014*).

**2)** Tumours that spontaneously regress have histological features similar to sympathogonia arrangements (*Beckwith* et *Perrin* – *1963*).

**3)** Mice with overexpression of N-myc proto-oncogene protein (MYCN) in neural crest progenitors spontaneously develop NB tumours (*Weiss* et al – *1997*).

**4)** NB cell lines are capable to differentiate into neurons or Schwannian cells, and they maintain the capacity to migrate via neural crest pathways and colonize different tissues (*Tomolonis* et al – *2018*).

**5)** Gene expression patterns of neuroblastoma cells are similar to neuro crest progenitors (*de Preter* et al – 2006; *Wylie* et al – *2015; Kholodenko* et al – *2018*).

### 1.2 Clinical presentation

Children with NB tumours present a great variety of symptoms related with the location of both primary and metastatic lesions. Most tumours appear in the abdomen, commonly in the adrenal glands and might cause hypertension, abdominal pain, distension, constipation, local effects on abdominal organs or might be asymptomatic (*Whittle* et al – *2017*). About one tenth of NB patients present tumour invasion into the epidural or the intradural space, resulting in spinal compression and paraplegia (*De Bernardi* et al – *2005*). Primary tumours that arise in the cervical or thoracic region might cause respiratory symptoms and Horner syndrome (alterations in eyelids) (*Whittle* et al – *2017*, *Matthay* et al – *2016*) (Figure 2).

Metastatic lesions in bone marrow, bone and regional lymph nodes, are found in about 50% of NB patients at diagnosis. These can be accompanied with bone pain, fever, weight loss, bleeding from anaemia and thrombocytopaenia. Liver involvement can cause liver disease in about 20-30% of NB patients, however metastasis in nervous system and lung are rare, present in less than 5% (*Fernandez-Pineda* et al – *2015*; *DuBois* et al – *1999*).
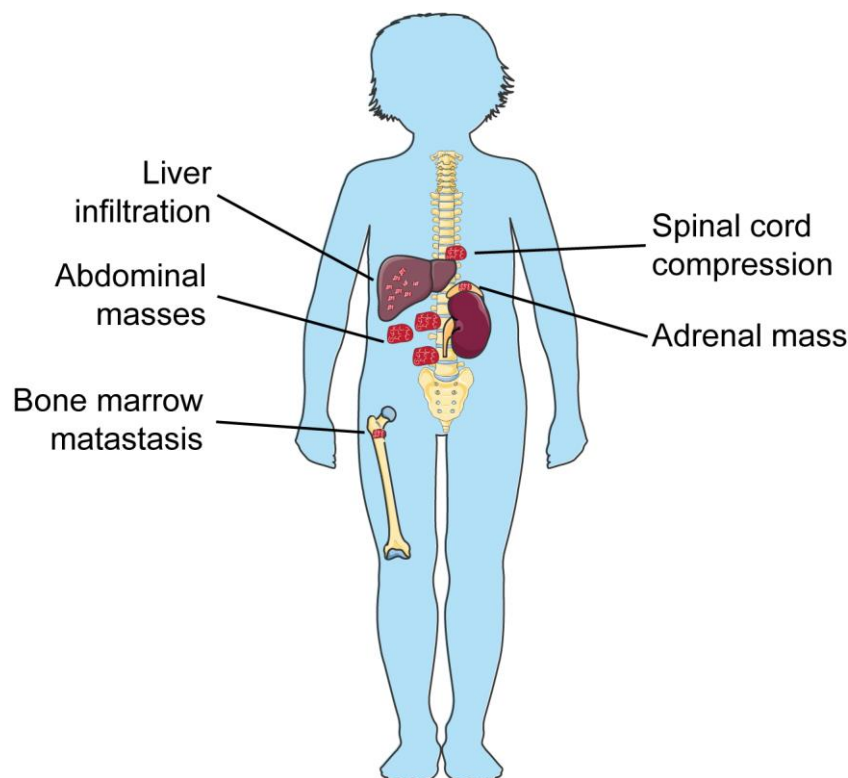
**Figure 2 – Locations of NB tumours and metastasis.** NB primary tumours are commonly presented as abdominal or adrenal masses. Depending on tumour location, organ function can be altered. One fifth of NB patients show lesions in the liver and in rare cases, lesions can be present close to the spinal cord compressing it, and in some cases causing paralplegia. The most common site of metastasis and relapse disease of NB is the bone marrow, often relapse of NB (adapted from *Maris* et al – *2010*).

## 1.3   Diagnosis and risk stratification of NB

Diagnosis of neuroblastoma is based on the presence of specific histopathological features in tumour tissue or presence of tumour cells in a bone marrow aspirate or biopsy, accompanied by raised concentrations of urinary catecholamine metabolites (dopamine, homovalinic acid – HVA and/or vanillymandelic acid – VMA) (Strenger et al – 2007). High-risk patients often have raised concentrations of serum lactate dehydrogenase, ferritin, or chromagranin, but these are relatively non-specific and are not independent predictors of outcome in light of modern biological co-variates (*Whittle* et al – *2017*, *Matthay* et al – *2016*). Risk assessment considers several clinical and biological features. Through the years there has been an attempt to homogenise risk stratification approaches to allow comparison of NB patients treated in several world treatment centers. The International Neuroblastoma Staging System (INSS) considers several surgical criteria such as degree of tumour resections, presence of lymph nodes involvement

and tumour infiltration across the midline. In addition, it distinguishes highly aggressive metastatic tumours (stage 4), metastatic tumours that spontaneously regress (stage 4S), as well as non-metastatic, loco-regional tumours (stage 1, 2 and 3) (Table 1) (*Brodeur* et al – *1988*). The INRG is a pre-surgical staging system, it uses imaging techniques to evaluate tumour morphology and identify image-defined risk factors (IDRF). These features allow radiologists to discriminate between locoregional tumours without involvement or surrounding tissue (stage L1) from invasive tumours (L2). Furthermore, highly metastatic tumours (stage M) are

| System | Stage | Criteria |
|--------|-------|----------|
| INSS | 1 | Localized tumour, grossly resected, no lymph node involvement |
| | 2A | Unilateral tumour, incomplete gross excision, negative lymph nodes |
| | 2B | Unilateral tumour with positive ipsilateral lymph nodes |
| | 3 | Tumour infiltrating across midline or unilateral tumour with contralateral lymph node or midline tumour with bilateral lymph nodes |
| | 4 | Distant metastatic disease |
| | 4S | Localized primary tumour as defined by stage 1 or 2 in patient under 12 months with dissemination limited to the liver, skin and/or bone marrow (<10% involvement) |
| INRG | L1 | Localized tumour with no image defined risk factors (Monclair et al – 2009) |
| | L2 | Localized tumour with one or more image-defined risk factors (Monclair et al – 2009) |
| | M | Distant metastatic disease |
| | MS | Metastatic disease in children under 18 months with metastases limited to skin, liver and/or bone marrow (<10% involvement) |

**Table 1 – Staging criteria for INSS and INRG (***Sokol* et al – *2019***)**

differentiated from metastatic cases that spontaneously regress (stage MS) (Table 2) (*Monclair* et al – *2009*).
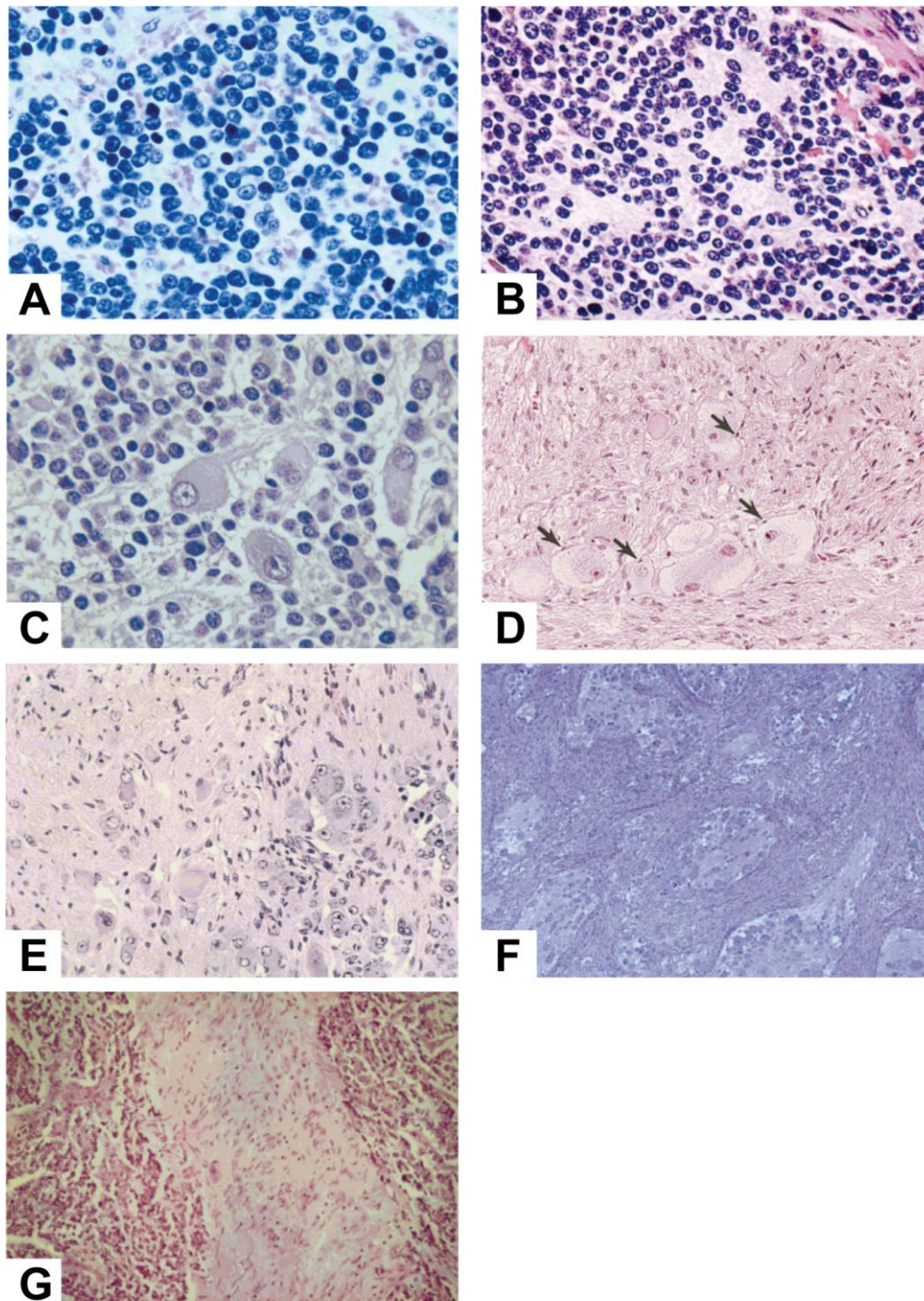
**Figure 3 – Representation of the diverse histology neuroblastic tumours**. **A –** Undifferentiated NB (original magnification x200); **B –** Poorly differentiated NB (original magnification x200); **C –** Differentiating NB (original magnification x400); **D –** mature Ganglioneuroma, arrows indicate mature ganglion cells (original magnification x200); **E –** Maturing Ganglioneuroma (original magnification x100); **F –** Ganglioneuroblastoma, intermixed (original magnification x40); **G –** Ganglioneuroblastoma, nodular (original magnification x100) (adapted from *Shimada* et al – *2000*).

The Shimada classification system was first developed in 1984 and it

considered several histological features to distinguish favourable versus unfavourable neuroblastic tumours, such as presence and degree of stroma, grade of differentiation, mitosis-karyorrhexis index (MKI), presence of nodules, and age. It

| Type | Subtype | Histological features |
|---|---|---|
| **Neuroblastoma** | Undifferentiated | • Small round cells with undiscernible to thin cytoplasm<br>• Nuclei with variable shape and granular chromatin |
| | Poorly differentiated | • Background of neuropil with variable length<br>• Less than 5% of tumour cells present cytomorphic feature of ganglion differentiation |
| | Differentiating | • Abundance of neuropil<br>• More than 5% show sign of ganglion cell differentiation<br>• Larger cytoplasm to nuclei ratio |
| **Ganglioneuroma** | Mature | • Composed of mainly ganglioneuromatous stroma<br>• Presence of scattered differentiating neuroblasts and/or ganglion cells |
| | Maturing | • Composed of mature Schwannian stroma and ganglion cells<br>• Absence of neuroblastomous in order to terminate maturation |
| **Ganglioneuroblastoma** | Intermixed | • Incomplete maturation with lingering foci of neuroblastic cells<br>• Ganglioneuromatous component to neuroblastic foci higher than 50% |
| | Nodular | • Visible hemorrhagic neuroblastic nodules together with ganglioneuroma or intermixed ganglioneurblastoma<br>• Neuroblastic nodules pushing into stroma-rich, demonstrating proliferative growth obstructing schwannian stroma |

**Table 2 – Histological features of neuroblastic tumours** (*Shimada* et al – *2000*).

was later revised to update the histological prognosis factors and establish the International Neuroblastoma Pathology Classification (INPC). INPC incorporates multiple factors consisting of diagnostic category (accounting for quantity of Schwannian stromal development and grade of tumour differentiation), MKI, and age to ultimately define tumour histology as favourable versus unfavourable (Table 2). Using these features, tumour can be classified into the following histology groups: Undifferentiated NBs, composed of small round tumour cells (Figure 2A), with a barely discernible cytoplasm or neuropil. Differentiating or differentiated NBs have cells with a larger cytoplasm with the presence or neuropils of different sizes (Figure 2B-C) (Shimada et al – 2000). Ganglioneuroblastomas are composed of more than half Schwannian stromal tissue, and dispersed neuroblasts (Figure 2D). In ganglioneuromas there is mature stroma, differentiating neuroblasts and maturing ganglion cells (Figure 2E).

An important biological feature to consider is patient age. Earlier studies observed that the younger patients showed better prognosis than the older ones, setting 12 months of age as the threshold between risk groups (*Breslow* et al – *1971*). However, retrospective studies in the early 2000s raised the question if this age division should be altered from 12 to 18 months of age (*London* et al -*2005*). Ten years ago, the International Neuroblastoma Risk Group (INRG) performed a retrospective study with large cohort of patients (11037 patients, enrolled on the Children's Oncology Group (COG)), and established a clinical cut-off age of 18-month-old.

All these staging systems together with biological features of the tumour (see in section 1.5) permit the risk stratification of NB patients in very low, low, intermediate and high-risk groups (Table 3) (*Cohn* et al – *2009*).

## 1.4 Biological features of neuroblastoma

Advances in NB research led to the identification of recurrent genetic and genomic aberrations associated with patient prognosis. Some are strong prognosis predictors, while others are indicative of differentiation status.

| Stage | Criteria |
|---|---|
| **Very low** | L1/L2; GN maturing or GNB intermixed |
| | L1; any histological diagnostic (except GN maturing or GNB intermixed) |
| **Low** | L2; <18 months, any histological diagnostic (except GN maturing or GNB intermixed); *MYCN* NA; no 11q alteration |
| | L2; ≥18 months, GNB nodular or differentiating neuroblastoma; *MYCN* NA; no 11q alteration |
| | M; <18 months; *MYCN* NA; Hyperdipliod |
| | MS; <18 months; *MYCN* NA; no 11q aberration |
| **Intermediate** | L2; <18 months, any histological diagnostic (except GN maturing or GNB intermixed); *MYCN* NA; 11q aberration |
| | L2; ≥18 months, GNB nodular or differentiating neuroblastoma; *MYCN* NA; 11q aberration |
| | L2; ≥18 months, GNB nodular or undifferentiated or poorly differentiating neuroblastoma; *MYCN* NA; |
| | M; <18 months, diploid |
| **High** | L1; any histological diagnostic (except GN maturing or GNB intermixed); *MYCN* A |
| | L2; *MYCN* A |
| | M; <18 months; *MYCN* A |
| | M; ≥18 months |
| | MS; *MYCN* NA; 11q aberration |
| | MS; <18 months, *MYCN* A |

**Table 3 – Risk stratification of NB patients** GN- ganglioneuroma, GNB – ganglioneuroblastoma, MYCN A – MYCN amplified, MYCN NA – MYCN non-amplified (Cohn et al – 2009).

### 1.4.1 Genetic alterations

*MYCN amplification* – Amplification of the oncogene v-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma (*MYCN)* was one the first prognosis markers described for NB. In the early 1980s it was described that NB cell lines and tumours with an undifferentiated phenotype presented amplification of the oncogene MYCN (Figure 4). Amplification of MYCN is the genetic aberration most consistently associated with patient poor outcome and is detected in ~20% of all NB

cases (*Bérnard* – 1995, *Huang* et *Weiss* – *2013, Brodeur* et al – *1984, Seeger* et al – *1985*). MYCN is a member of the MYC family of transcriptions, which are key regulators of critical cellular processes, including survival, proliferation, and
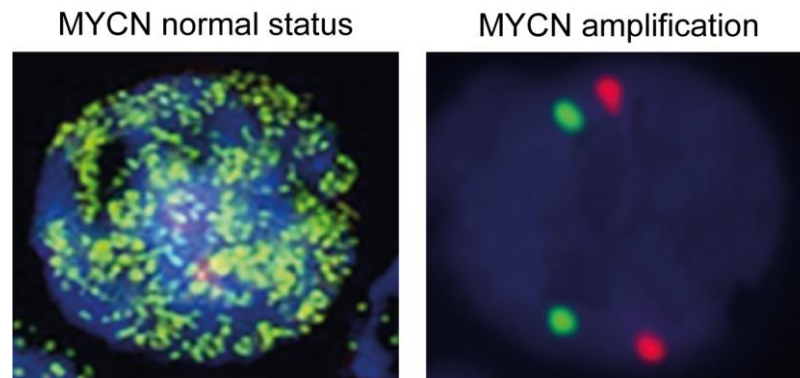


**Figure 4 – MYCN amplification is an indicator of high-risk NB patient's prognosis.**
Representation of amplification of the MYCN gene. Green signal is the probe for MYCN and red signal is the probe for CEP2 internal control (adapted from *Wang* et al – *2013*).

differentiation, many of which are related to tumour initiation and progression. (*Huang* et *Weiss* – *2013*).

MYCN is expressed during early developmental stages but not present in adult tissues (*Huang* et *Weiss* – *2013*). MYCN is a member of the MYC family of transcriptions, which are key regulators of critical cellular processes, including cell survival, proliferation, regulation of differentiation, angiogenesis, migration and invasion, immune surveillance and apoptosis (*Huang* et *Weiss* – *2013*). NB cell lines with amplification of MYCN demonstrate metastatic behaviour and altered cell adhesion, motility, invasion and migration (*Bérnard* – *1995, Huang et Weiss* – *2013*). Particularly MYCN was found to inhibit the expression of integrins α1 and β1 promoting cell detachment and increasing cell migration and metastasis (*Huang* et *Weiss* – 2013). Furthermore, amplification of MYCN induces the secretion of vascular endothelial growth factor by activating the PI3K/mTOR pathway (*Kang* et al – *2008*). Inhibition of this pathway *in vivo* reduces tumour growth and angiogenesis (*Chanthery* et al – *2012*). MYCN up-regulates the expression of the polycomb and self—renewal protein BMI1 while also inhibiting cell differentiation (*Huang* et *Weiss* – 2013). Moreover, amplification of *MYCN* is associated with T-cell poor microenvironment and inhibits processes of antigen presentation, such as the secretion monocyte chemoattractant protein-1 (MCP-1) (*Song* et al – *2007; Layer* et al – *2017*). Tumour suppressor gene *TP53* is rarely altered in NB, however its

activity is regulated by *MYCN* and *MDM2* (*He* et al – 2014). MDM2 stabilizes *MYCN* mRNA levels, and in turn, this oncogene also regulates MDM2. The feedback loop p53/MDM2/MYCN helps to maintain a proliferative and anti-apoptotic state of NB cells (*Ruiz-Pérez* et al – 2007). Additionally, treatment of neuroblastoma cell lines with differentiation inducers (such as retinoids) reduces its expression (*Huang* et *Weiss* – 2013).

ALK alterations – ALK, encoding the anaplastic lymphoma kinase receptor, is the most common somatically mutated gene in NB, with mutations present in around 9% of primary NB tumours and approximately 14% in the high-risk NB. Gain-of-function mutations in anaplastic lymphoma kinase (ALK) have been described in familial NB (1-2% of NB cases), (*Mossé* et al – 2008) Focal amplification of ALK is reported in 1–2% of NB cases and is mutually exclusive of point mutation. ALK amplification is almost exclusively associated with co-amplification of MYCN. ALK alterations confer poorer prognosis for tumours in the intermediate- and high-risk groups (*Bresler* et al – 2014).

The ALK receptor plays a role in the development of sympathoadrenal lineage of neural crest cell proliferation and differentiation (*Souttou* et al – 2001, *Motegi* et al – 2004). Activating *ALK* mutation F1174L, although sporadic, contributes for NB tumorigenesis in mice where MYCN is overexpressed (*Berry* et al – 2012). Recently, high expression of *ALK* was associated with high expression of *MYCN* and unfavourable prognosis (*Chang* et al – 2020). Basis for cooperation between ALK and MYCN might be due to ALK-mediated activation of PI3K signalling, inducing stabilization and increased levels of MYCN (*Zhu* et al – 2012). ALK inhibitors have been tested in preclinical models and some reached clinicals trials. However, they were dropped either by toxicity or lack of efficacy (*Janoueix-Lerosey* et al – 2017). Additionally, most promising results showed induced drug resistance mutations. Recent studies attempt to find combination of drugs that target both ALK and its downstream pathways (*Janoueix-Lerosey* et al – 2017).

*LIN28B* – The RNA binding protein *LIN28B* inhibits the let-7 family of microRNAs preventing its maturation. During embryogenesis Lin28B regulates the expression of Let-7 and controls proliferation during neural crest linage commitment. Downregulation of *LIN28B* inhibits cell proliferation and increases cell differentiation. Recently it has been shown to regulate the stability of the Aurora kinase A and the GTP-binding nuclear protein RAN (*Molenaar* et al – 2012; *Schnepp* et al – 2015).

Transgenic mice with LIN28B overexpressed in neural crest cells develop tumours with biological features similar to NB tumours (*Molenaar* et al – *2012*). Recently, it was demonstrated that LIN28B is an important regulator in NB metastasis by a mechanism involving MYCN and PDZ binding kinase (PBK), an enzyme that promotes self-renewal and migration of neural stem cells (*Chen* et al – *2020*).

PHOX2B alterations – Studies performed in familial NB, representing about 2% of all NB cases, identified paired-like Homeobox 2B (PHOX2B), as the first bona fide gene that can predispose to NB when mutated in the germline. PHOX2B is associated with proliferation and differentiation of human neuroblastoma cells. PHOX2B expression is specific for NB in its differential diagnosis with other small round cell tumours, as well as a specific biomarker for both bone marrow NB infiltration and minimal residual disease (MRD) (*Stutterheim* et al – *2008, Burchill* et al – *2017*).

PHOX2B is a transcription factor essential for embryonic development, as PHOX2B knockout mice die at embryonic day 14, unless they are supplied with noradregenic agonists (*Thomas* et *Palmiter* – *1997*). This protein was shown to be important during cell cycle exit by neural crest cells and it participates in neuronal differentiation (*Rohrer* – *2011*). Furthermore, differentiation of NB cell lines with retinoic acid decreased PHOX2B levels (*Yang* et al – *2016*).

Other genomic alterations – Several whole-genome studies of NB tumours have been accomplished to characterize recurrent alterations associated with NB prognosis and clinical evolution. Overall, NB is characterized by a low somatic mutation count, with few recurrent mutations beyond those found in ALK. Cheung and colleagues found ATRX (encoding the RNA helicase, transcriptional regulator ATRX) loss-of-function mutations and deletions associated with NB in adolescents and young adults. Loss-of-function mutation of *ATRX* and promoter rearrangements of *TERT* (encoding telomerase reverse transcriptase) are associated with the activation of the alternative lengthening of telomeres (ALT) pathway in high-risk NB (*Ackermann* et al – *2018*). These alterations were found in 11% and 25% of NB patients, respectively, and were not present in tumours with *MYCN* amplification (*Valentijn* et al – *2015*). Moreover, dysregulations in chromatin remodelling genes were described in high-risk NB patients, including chromosomal deletions and sequence alteration in the Polycomb complex genes *ARID1A* and *ARID1B*, associated with treatment failure and poor patient's survival (*Sausen* et al – *2013*).

### 1.4.2 Chromosomal alterations

Clinical course of NB is greatly influenced by the presence of copy number variations (CNVs). Numerical CNVs are observed with gain or loss of entire chromosomes, whereas structural CNVs include gains and losses of partial chromosomes. Localized tumours (low-risk NB) display several numerical CNVs, whereas, aggressive metastatic tumours are characterized by a number of structural recurrent CNVs, including deletions of the 1p, and 11q, and gain of chromosome arm 17q (*Janoueix-Lerosey* et al – *2009*).

Loss of 1p (short arm of chromosome 1) – Loss of chromosome 1p was first described as a poor prognosis feature by Caron and colleagues. In addition, they found that it was associated with aggressive disease and poor response to treatment in patients from lower NB stage groups (*Caron* et al – *1996*). A large COG cohort further demonstrated the association of loss of 1p with poor prognosis factors, such as unfavourable histology, amplification of MYCN, INSS stage 4 and patients older than 12 months (*Attiyed* et al – *2005*).

Loss of chromosome 11q (long arm of chromosome 11) – More than one third of primary NB tumours shows loss of 11q. This alteration is associated with high-risk tumours features, but it is seldom found in MYCN amplified tumours (*Guo* et al – *1999*).

Other chromosomic alterations – Common segmental chromosomal alterations include loss of 3p, 4p and 14q and gains of 1q, 2p and 17q. Loss of heterozygosity of chromosomal region 3p was found in 16% of neuroblastomas, and it was related with the loss of a tumour suppressor locus (*Hallstensson* et al – *1997*; *Ejeskä*r et al – *1998*).

Gain of chromosome arm 17q is the most frequent cytogenetic abnormality of NB cells, found in 46% of cases. This gain has been associated with high-risk features, such as loss of 1p and MYCN amplification, and observed more frequently in advanced stages of the disease or older children. However, the prognostic relevance of chromosome 17q gain in NB is still discussed (*Brown* et al – *1999*). In low stage patients a non-altered chromosome 17 or partial gain of 17q is an independent indicator of poor patient prognosis (*Vandesompele* et al – *2005*).

The biological relevance of these chromosomal alterations is still being studied. However, a few are known to alter the expression levels of tumour suppressor gene

and oncogenes important for neuroblastoma tumorigenesis (*Westermann* et al – *2002*).

### 1.4.3  Differentiation markers

Neuroblastic tumours include different histological subtypes characterized by diverse proportions of Schwann cells and neuroblasts, that can be distinguished by their degree of cellular differentiation and maturation. NB, stroma poor undifferentiated tumour, is the most malignant in nature. Despite its undifferentiated histology, a portion of NB tumours occasionally mature or regress spontaneously (stage 4S), while part retain the capacity to undergo neuronal differentiation under specific treatment conditions (differentiation agents/therapy)

Trompomyosin receptor kinases (Trks): Tropomyosin kinase receptors are part of a receptor family of tyrosine kinases coded by *NTRK* genes.  Trks are involved in the regulation of neuronal plasticity and development (*Huang* et *Reichardt – 2003; Barbacid – 1994*). Trk receptors (TrkA, TrkB and TrkC) differ in their extracellular domain, conferring ligand specificity (*Brodeur* et al – *2009*). Each Trk can be activated by one or more of neurotrophin-nerve growth factor (NGF), brain-derived
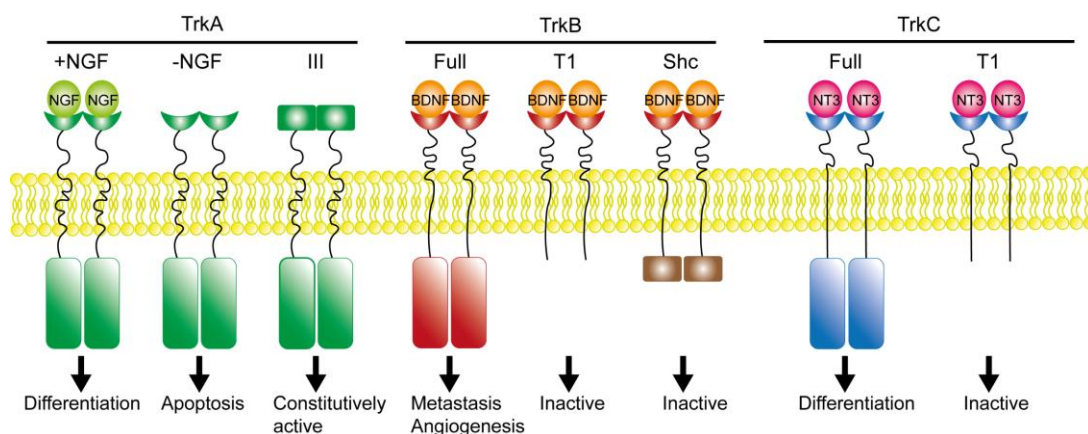


**Figure 5 – Schematic representation of Trk receptors and common isoforms.** Trk receptors differ in their extracellular domains which confers them with specificity for different ligands and activation of different intracellular pathways. Activation of TrkA with NGF activates pathways inducing cell differentiation, in the absence of this neurotrophin different pathways are activated inducing cell apoptosis. Isoform III of TrkA has a constitutively active extracellular domain. TrkB is activated by BDND, inducing metastasis and angiogenesis of tumour cells. This receptor has two inactive isoforms, one lacking the intracellular domain (T1) and another includes the SHC binding site (Shc). TrkC is also a differentiation inducer receptor, when activated by NT3. Although there are several truncated versions of this receptor the most predominant is T1, lacking the intracellular domain (adapted from *Brodeur* et al – *2009*).

neurotrophic factor (BDNF) and neurotrophins 3 and 4 (NT3, NT4) (Figure 5). However, alternative splicing may alter these interactions (*Amatu* et al – *2019*) (Figure 5). Trk signalling plays a critical role in cell survival, proliferation, fate of neural precursors, axon and dendrite growth, and activation of ion channels and neurotransmitter receptors. In the adult nervous system, the Trk receptors regulate synaptic plasticity. Knock-out mice for Trk receptors show abnormal development of the central and peripheral nervous system.  Particularly, TrkB deficiency causes the most severe phenotype with mice having abnormal movements and posture (*Smeyne* et al – *1994*; *Klein* et al – *1993*; *Klein* et al – *1994*).

In NB, each receptor plays an important role in the diverse behaviours of the disease, Expression of TrkA and TrkC are associated with biologically favourable NB tumours, prone to spontaneous regression and differentiation. In contrast, TrkB expressing tumours are associated with aggressive and high-risk disease. (*Brodeur* et al – *2009*). Overexpression of TrkA in the NB cell line SK-N-SY5Y reduced tumorigenesis and expression of pro-angiogenic factors, like VEGF, Ang2, bFGF, while overexpression of TrkB had the opposite effect (*Egger* et al – *2002*). This depends on the expression of the different Trks and the presence or absence of their ligands in the microenvironment, leading to the activation of different intracellular pathways (*Huang* et *Reichardt – 2003*). Treatment of low stage tumours with NGF, TrkA ligand, induces cellular differentiation. whereas in more aggressive tumours where expression of TrkA is very low, cells do not respond to NGF treatment (*Nakagawara* et *Brodeur GM – 1997*). The TrkB ligand (BDNF) is highly expressed in high-risk NB and it increases the metastatic and invasion potential of NB cell lines (*Nagakawara* et al – *1994, Matsumoto* et al – *1995*). Expression of TrkC is high in NB with favourable prognosis, younger patients with low stage tumours without amplification of *MYCN* (*Yamashiro* et al – *1996*). However, its ligand, NT3, was also found increased in aggressive NBs and blockage of the NT3/TrkC interaction induced apoptosis and inhibited tumour growth (*Bouzas-Rodriguez* et al – *2010*).

Nerve Growth Factor Receptor (NGFR): NGFR also denominated Low Affinity Neurotrophin Receptor p75NTR, regulates differentiation and survival of neuronal populations during development (*Huang* et *Reichardt – 2003*).  In NB, NGFR is highly expressed in tumours with clinical and biologically favourable features, such as young patients, differentiated histology and *MYCN* non-amplified tumours. In addition, patients with expression of NGFR show a longer EFS and OS (*Schulte* et al

– *2009*). In NB cell lines, overexpression of NGFR reduces its proliferative capacity and increases apoptosis. Additionally, overexpression of NGFR reduced *in vivo* tumorigenicity of NB cell lines by driving cells to a more differentiated and benign phenotype (*Schulte* et al – *2009*).

Neurofilament high peptide (NEFL): NEFL is one of the three subunits of the neurofilament proteins expressed in neurons, which play a central role in the transport of neurotransmitters throughout axons and dendrites and contribute to neuronal cytoarchitechture (*Leermakers* et *Zhulina* – *2010*). Overexpression of NEFL in NB cell lines reduced cell proliferation and enhanced differentiation. Furthermore, high expression of NEFL in NB tumours was correlated with better patient survival, more differentiated histology and lower tumour stages. No association was made between levels of NEFL and age of the patients, status of *MYCN* and risk stratification (*Capasso* et al – *2014*).

Tubulin beta 3 (Tubβ3): Tubβ3, coded by the gene *TUBB3*, is a member of the beta tubulin protein family. This protein is primarily expressed in neurons and was shown to be important for the development of neurite growth. Tubβ3 knock-down in NB cell lines prevented retinoic acid response. In addition, these cells were resistant to DNA damage by free radicals and reactive oxygen species (*Guo* et al – *2010*). Expression of Tubβ3 was present in all types of neuroblastic tumours, and more recently it was shown its importance in resistance to microtubule targeting drugs, commonly used to treat NB (*Dráberová* et al – *1998*; *Don* et al – *2004*).

Chromodomain helicase DNA binding 5 (CHD5). CHD5 gene encodes a member of the chromodomain helicase-DNA binding family. CHD5 regulates the structure and unfolding of chromatin during neurogenesis (*Egan* et al – *2013*). It is mapped in the chromosome 1p, often deleted in NB, and its expression is low or absent in aggressive NBs (*Koyama* et al – *2012*). Increase of *CHD5* in NB cell lines, either using plasmids or demethylating drugs, inhibited *in vitro* and *in vivo* tumorigenicity (*Fujita* et al – *2008*; *Li* et al – *2012*).

CD44: CD44 is a surface glycoprotein important for cell adhesion and cell-to-cell interaction, which was shown to have different roles in several tumours (*Chen* et al – *2018*). In NB, its expression is absent in more aggressive NBs and associated with loss of TrkA expression (*Kramer* et al - 1997). Treatment of NB cell lines with differentiation agents increased the expression of CD44 (*Gross* et al – *1995*).

| Marker | Prognosis | Mechanism | References |
|--------|-----------|-----------|-----------|
| TrkA | Good | • Reduces expression of angiogenic factors<br>• Overexpression reduced tumorigenic potential of NB cell lines | *Eggert* et al – *2002*; Lavoie et al – 2005; |
| TrkB | Poor | • Overexpression increases aggressiveness and tumorigenic potential of NB cell lines<br>• Activation with BDNF increases metastatic and tumorigenic potential | *Eggert* et al – *2002*; *Nagakawara* et al – *1994; Matsumoto* et al – *1995* |
| TrkC | Good | • Disruption of NT3/TrkC activation increases cell apoptosis and inhibits tumour growth | *Yamashiro* et al – *1996; Bouzas-Rodriguez* et al – *2010* |
| NGFR/ p75 | Good | • Inducer of apoptosis and differentiation in NB cell lines<br>• Inductor of NB differentiation and reduced *in vivo* tumorigenesis | *Eggert* et al – *2000*; *Schulte* et al – *2009* |
| NEFL | Good | • Inhibitor of tumorigenesis and invasion | *Capasso* et al – *2014* |
| S100β | Good | • Glial cell marker<br>• Expression associated with better prognosis in unfavourable tumours | *Spreca* et al – *1989, Van Eldik* et *Zimmer* – *1988; Nagoshi* et al – *1992* |
| Tubβ3 | Good | • Central player in neurite growth and neurodifferentiation<br>• Important role in chemoresistance | *Guo* et al – *2010; Don* et al – *2004* |
| CD44 | Good | • Low to absent expression in aggressive NB<br>• Expression induced by differentiating agents | *Gross* et al – *1995; Kramer* et al – *1997* |
| CHD5 | Good | • Methylation of promoter in aggressive NBs<br>• Induction of expression reduces NB cell line tumorigenesis | *Fujita* et al – *2008*; Li et al – 2012; Koyama *et al* – 2008 |

**Table 4 – Mechanisms of differentiation markers of neuroblastoma**

Additionally, transfection of CD44 into NB cell lines increased its adherence capacity, unless they have MYCN amplification, which alters CD44 functionality (*Gross* et al – *2000*).

S100 Calcium Binding Protein B (S100β). S100β is a calcium binding protein, highly expressed in the nervous system and is a well-established glial cell marker (*Spreca* et al – *1989, Van Eldik* et *Zimmer* – *1988*). Knowledge about the role of S100β in neuroblastoma is scarce. However, expression of this calcium protein is associated with good prognosis in unfavourable NBs (*Nagoshi* et al – *1992*).

## 1.5  Current NB therapy and advances

Intensity and protocol of therapy used to treat NB patients depends on the assigned risk group. Treatment of NB can include observation only, surgery, chemotherapy, radiotherapy, myeloablative chemotherapy, differentiation therapy and immunotherapy.

Low-risk NB – Low-risk patients account for 50% of all new diagnosed NB and show survival rates higher than 90%, with minimum or no therapy including surgery. Localized adrenal tumours with favourable biological features are closely observed only and the majority spontaneously regress with child's growth (*Nuchtern* et al – *2012*). The need for treatment arises depending on tumour location and symptoms associated (spinal cord injury or lung compression). In this case, they undergo surgical excision and mild chemotherapy in the case of relapse (*Monclair* et al – *2015*). Metastatic stage 4S/MS tumours show favourable biological features, and in most cases, they spontaneously regress with little to no chemotherapy (*Brodeur* et al – *2018*). Patients receive treatment (surgery or chemotherapy) when there is risk of complications and death: large hepatomegaly or large tumours causing mechanical obstruction, liver disfunction or respiratory distress (*Whittle* et al – *2017*).

Intermediate-risk NB – Intensity and length of therapy of intermediate risk patients depends on tumour biological features. Differentiation between favourable and unfavourable cases allows for reduction in treatment and associated toxicities, while maintain high survival rates (88% and 96% 3-year OS). In addition to chemotherapy, surgical resection of the residual primary tumour is performed when possible. However, for a reduced percentage of patients this course of treatment is not enough. In these cases, radiotherapy might be necessary (*Baker* et al – *2010*).

High-risk NB – High-risk patients are about half of the cases of NB. In spite of the complexity of the intensive multimodality treatment approaches, survival rate of these patients remains lower than 50%. COG and OPEC/COJEC protocols are the most common for treating NB patients and several groups use variations of these protocols. Both include four phases: induction chemotherapy, local control, consolidation and maintenance therapy (*Whittle* et al – *2017*) (Figure 4).

1.    *Induction chemotherapy* – The main objective of this stage is to reduce tumour burden by shrinking the primary tumour and reducing metastasis. In all protocols, induction is the most intense phase with a combination of anthracyclines (doxorubicin), alkylating agents (cyclophosphamide, cisplatin, melphalan), mitotic inhibitors (vincristine) and topoisomerase inhibitors (irinotecan, topotecan, etoposide). Most protocols use these chemotherapy drugs; however, the number of cycles and the drug combination differs between protocols and institutions. Patients that do not respond to this high intensity treatment schedule (about 20%) have a very poor prognosis (*Whittle* et al – *2017*; *Matthay* et al – *2016*).

2.      *Local control* – Surgical resection and radio beam therapy of localized lesions are used to prevent local recurrences and are performed during the induction stage (between cycles 4 and 6). Surgical tumour resection is often accompanied with radiotherapy of the primary tumour site, resulting, in better local control and OS (*Du* et al – *2014; Mazloom* et al – *2014*).
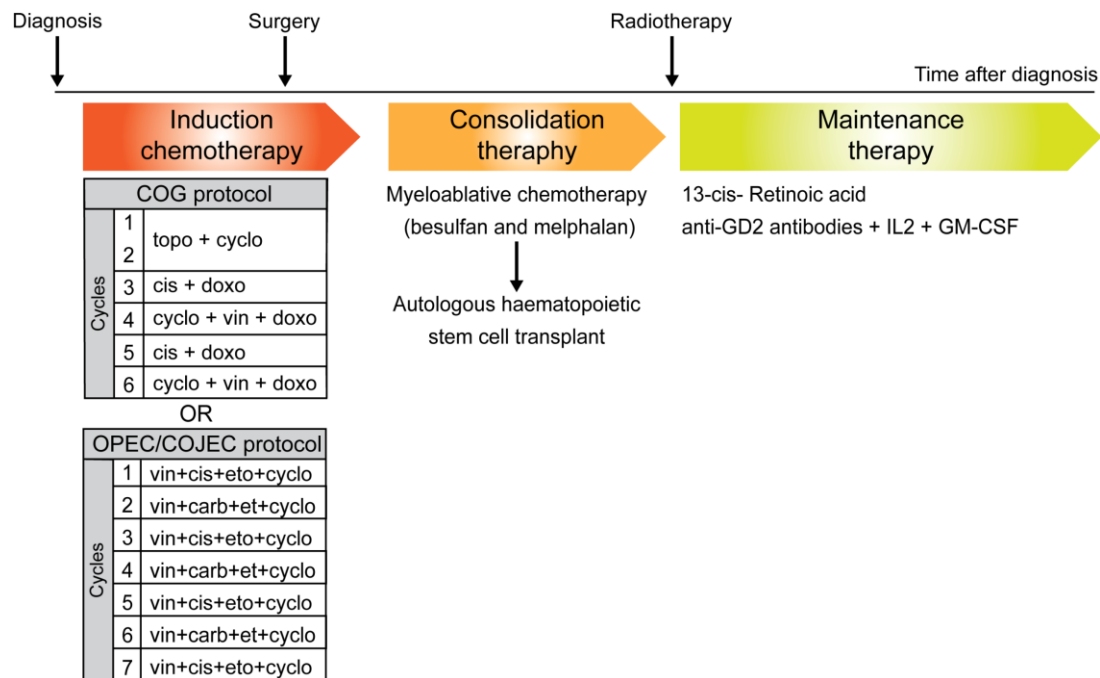


**Figure 6 – Therapeutical strategies for high-risk NB patients.** Diagnosis and stratification of NB patients is done by a multi-disciplinary team. High-risk patients undergo a high intensity treatment schedule that consists of Induction chemotherapy, consolidation therapy and maintenance therapy. Effectiveness of treatment during induction chemotherapy can define patient's survival. There are two different treatment protocols used during the first phase, composed of the same drugs but used in different schedules: topotecan (topo); cyclophosphamide (cyclo); doxorubicin (doco); cisplatin (cis); vincristine (vin); etoposide (eto). During induction patients may undergo surgery for the local control of tumours and improve chemotherapy efficacy. Consolidation therapy involves myeloablative chemotherapy followed by autologous haematopoietic stem cell transplant. In addition, after this stage patients may receive radiotherapy for control of lesions. Patients that enter remission, receive maintenance therapy consisting of 13-*cis*-RA alone or in combination with anti-GD2 antibodies, IL-2 and GM-CSF.

3.      *Consolidation treatment* – Patients that respond to induction treatment and local control, undergo consolidation phase. Although in many protocols this stage consists in myeloablative chemotherapy followed by autologous stem cell rescue, the efficacy of this treatment has been questioned and some institutions no longer perform it (*Whittle* et al – *2017*, *Kushner* et al – *2016*).

4. *Maintenance therapy* – High-risk NB patients which enter in remission after consolidation treatment, receive maintenance therapy to eliminate residual tumour cells and prevent relapse, which often are more aggressive and chemo resistant tumours (*Whittel* et al – *2017*). Maintenance phase of therapy includes immunotherapy and a differentiating agent. For many years, isotretinoin (13-*cis*-retinoic acid (13-*cis*-RA)) was the main drug used during this phase, due to its differentiation capacities and its efficacy in improving EFS of NB patients (*Matthay* et al – *1999, Matthay* et al – *2009*). In the last 10 years, immunotherapy with anti-GD2 antibodies has proven to be effective in increasing EFS and OS of high-risk NB patients (*Cheung* et al – *1998; Cheung* et al – *2012; Kushner* et al – *2018*).

Despite the improvement in NB research, there are still high-risk patients that do not respond to induction therapy or relapse after/during maintenance treatment. Therapeutical approaches to treat relapse patients often involve surgery and multiagent chemotherapy combinations (*Zage* – *2018, Saylors* et al – *2001, Kushner* et al – *2010; Kushner* et al – *2006; Garaventa* et al – *2003*). New therapies are being developed using surface markers, such as norepinephrine transporters, CD171, NY-ESO-1, as drug delivery systems or immunotherapy targets (*Matthay* et al – *2007, Kholodenko* et al – *2018*).

## 1.6 Preclinical models of neuroblastoma

Knowledge about the origin of NB, and the evaluation of the effectiveness of new treatments came not only for retrospective patient analysis but also from the use of preclinical models, such as cell lines, xenograft mouse models and transgenic mouse models. NB cell lines are the most common model for the evaluation of new therapeutic approaches due to its availability and ability to grow indefinitely *in vitro*. These models show the same alterations as their original tumours (such as MYCN amplification, loss of 1q, etc). However, they acquire new alterations to become immortalized (*Thiele* – *1998*). Differentiation capacity of NB cell lines is maintained, making them a good model to study the molecular biology of neurogenesis and neuronal disease (*Shastry* et al – *2001*). In addition, there are drug resistant cell lines as well as drug sensitive cell lines, since tumour samples were collected at different treatment stages. This allows not only to evaluate the effect of different drug combinations for resistant tumours, but also to elucidate the underlying molecular mechanisms (*Shastry* et al – *2001*). Characterization of well-established NB cell lines

by microarrays, RNA sequencing for mRNA and DNA methylation profiling, allowed for a better understanding of the different cell responses (*Harenza* et al – *2017*).

Tumours comprise a complex network of cells and stroma, this intra-tumour heterogeneity is complicated to replicate *in vitro*. Recently, NB patient derived organoids (PDOs) were developed to recreate tumour complexity and a more faithful *in vitro* preclinical model. It was demonstrated that PDOs had the same mitotic index, genetic features and expression of stem cell markers as the primary tumours. Most importantly, they maintained tumour cell heterogeneity and cell viability after cryopreservation, making them a useful *in vitro* model (*Fusco* et al – *2019*).

Increased of knowledge about tumour biology has led to the development of new and improved preclinical models. These models aim to meet the following criteria: a) Resemble histopathological features of human tumours, such as cell morphology, aggressiveness and metastatic profiles; b) engraftment or tumour appearance should happen in short time and with high penetration rate; c) Cancer-host interaction similar to human tumours; d) immune response should be similar to the human tumours (*Kiyonari* et *Kadomatsu* – *2015*). There is an attempt to reduce the differences between tumour models and human tumours, however not all of these criteria are met. Cell line derived xenografts probably meet the least amount of the above-mentioned criteria. In this model, NB cell lines are injected into immunocompromised mice, and depending on tumorigenicity of the cell lines used, generation of tumours occurs weeks after cell inoculation. Cell line xenografts can help to predict the clinical relevance of a drug and are easily reproducible. However, there is no cell heterogeneity in the tumours generated and cultured cell lines acquire alterations that are not found in patient tumours (*Brakeveldt* et al – *2015*). Patient derived xenografts (PDX) models recapitulate better tumour heterogeneity, since they are generated from small fragments of patient's tumours implanted subcutaneously in immunocompromised mice. Serial passages maintain tumour's key molecular features; however, subcutaneous environment is different from tumour's original location, and can alter tumorigenic and metastatic capacities of tumour cells (*Tentler* et al – *2012*). This limitation is bypassed in patient derived orthotopic xenografts (PDOXs), where NB tumour fragments are implanted in the adrenal grands. In this model, tumours have histological features similar to original tumour that are maintained unaltered across passages (*Brakeveldt* et al – *2015*). In addition, parent tumour microenvironment features are maintained in PDOXs, those

include rich tumour blood and lymphatic vascularization, presence of tumour macrophages and pericyte coverage (*Brakeveldt* et al – *2016*). Recently, the same group showed that PDOXs maintained RNA and protein expression patterns across passages and relative to their parent tumour (*Brakeveldt* et al – *2018*).

Genetically engineered mouse models were important to understand origins of NB and test therapeutic approaches in disseminated tumour models (*Kiyonari* et *Kadomatsu* – *2015*). One of the first models generated was the TH-*MYCN*, where the expression of MYCN is under the tyrosine hydroxylase promoter (TH), which is a gene expressed in PHOX2B positive cells of the sympathoadrenal lineage. In this model, mice develop tumours in the sympathetic ganglia (small thoracic tumours or abdominal masses) with histological features similar to NB (*Weiss* et al – *1997*). TH-*MYCN* model is the most commonly used transgenic model, due to its high penetration rate, short-term tumour development time, clinical and biological features resembling high-risk *MYCN* amplified tumours (*Kiyonari* et *Kadomatsu* – *2015*). This model was combined with other transgenes, such as *TP53* haploinsufficiency mouse model or capase-8 (Casp8) deficient mice. TH-*MYCN/TP53* haploinsufficient mice were shown to have a more oncogenic phenotype, with lower survival rates and chemoresistant tumours (*Chesler* et al – *2008*). These mice are good models to study relapse and refractory tumours, as this alteration is often present in relapse patients but not in pre-treatment tumours (*Kim* et *Shohet* – *2009*). Deletion of casp8, protein involved in cell apoptosis often deleted in *MYCN amplified mice*, was done under the TH promoter in TH-*MYCN* mice (*Teitz* et al – *2000*). These mice had more bone marrow metastasis than TH-*MYCN* mice, however there was no difference in tumour latency or overall survival of the models (*Teitz* et al – *2013*).

# 2    Retinoids

Retinoids, the active metabolites of vitamin A, are involved in the regulation of several cell functions and processes. They exert an important role during embryonic development in tissue differentiation but are also important for brain function and reproductive system homeostasis (*Niederreither* et *Dollé – 2008*). In clinical practice, their differentiation capacities make them interesting drugs to be used as cancer therapy agents, and 13-*cis*-RA is part of the treatment schedule of NB patients (*Whittle* et al – *2017*). Moreover, they show a wide range of dermatologic applications, including psoriasis, acneiform and keratinizaion disorders (*Orfanos* et al – *1997*). In this chapter it will be discussed the origin of retinoids and a few of the different isoforms, their mechanism of action and how they work as differentiation agents. In addition, it will be described what is known about retinoids as treatment of NB and known resistance mechanism.

## 2.1   Origin and isomerisation of retinoids

Retinoids are obtained via dietary intake of vitamin A (eggs, dairy and fish oils) or provitamin β-carotene (plant origin). Retinol is absorbed by the intestinal mucosa cells where it is esterified to retinyl esters, this compound is transported by the lymphatic system to the liver for storage. Mobilization of retinoids to other tissues occurs via oxidization of retinol to retinal or retinoic acid by retinaldehyde dehydrogenase, an essential enzyme for embryonic development (*Alltucci* et *Gronemyer – 2001; Niederreither* et al – *1999*).

Isomerization of retinoids is an important process as each retinoid has a different affinity to specific receptors affecting signal transduction (*Armstrong* et al – *2005*).  The main signalling retinoid is the isoform all-trans-retinoic acid (ATRA), however the isomers 13-*cis*-RA and 9-*cis*-RA are commonly used for therapeutic purposes (Figure 7). Generation of these different isoforms of retinoic acid occurs both due to intracellular biological processes and to sensitivity to environmental agents, such as light, air or excessive heat (*Barua* et *Furr – 1998*). Isomerization of retinoids in biological fluids is catalysed by compounds containing thiol-groups. In addition, it was described that in cell culture media independently of the initial retinoid used, there was the presence of ATRA, 13-*cis*-RA and 9-*cis*-RA (*Lanvers* et al – *1998*). Isomerisation of 13-*cis*-RA to ATRA was found to be mediated by gluthathione S-transferases, but it is unknown whether this reaction is a specific or

unspecific reaction (*Chen* et *Juchau – 1997*). An important aspect to consider in retinoid research it the photoisomerization of retinoids, which may alter its dosage, thus it is important to use cyclodextrins in the vehicle (*Matthay* et al – *2009*).
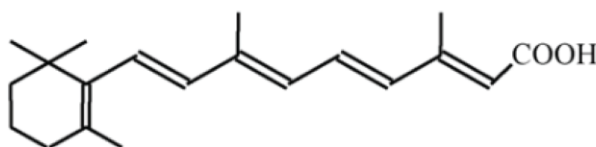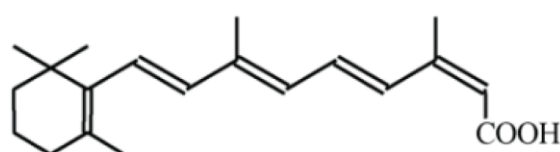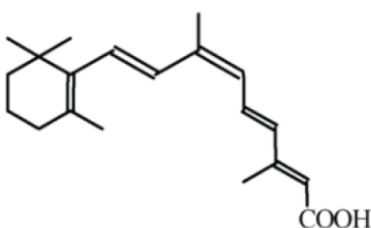


**Figure 7 – Chemical structure of three retinoid isomers** (adapted from *Armstrong* et al – *2005*)

## 2.2   Intracellular processing and transcription regulation

Membrane transporters are responsible for the uptake of retinol by the target cell. In the cytosol, retinol binds to cellular retinol binding proteins (CRBPs) an it is converted to retinaldehyde by alcohol dehydrogenase (ADH) or retinol dehydrogenase (RDH). Active forms of retinoid (ATRA, 13-*cis*-RA or 9-*cis*-RA are catabolized by retinaldehyde dehydrogenase 1, 2 and 3 (RALDH 1-3) and are transported into the nuclei. Here, they signal via retinoid receptors. Alternatively, they can be degraded by cytochrome p450 26 (CYP26) enzymes (*Green* et al – *2016*) (Figure 8).

Signalling of retinoids occurs via the binding of these molecules to two different classes of receptors, the retinoid acid receptor (RAR) and retinoid X receptor (RXR). Each of these receptors has three subtypes (α, β and γ) encoded by

different genes. Each subtype has different isotypes which use different promoters and alter the N-terminal domain (*Connolly* et al – *2013; Alltucci* et *Gronemyer – 2001*). Structure of RARs and RXRs is composed by a variable N-terminal domain, a DNA binding domain and ligand binding C-terminal domain. Ligand binding domain has as conformational flexibility of the C-terminal α-helix that has a different conformation in each subtype. The central DNA binding domain has conserved sequences important for both DNA binding and receptor dimerization (*Bastien* et *Rochette-Egly – 2004*). The most variable domain of retinoid receptors is the N-terminal that differs between types and subtypes and it was found to have phosphorylation sites that might be important for receptor flexibility (*Tanoury* et al – *2013*). RARs activity is induced by its ligands and by heterodimerization with RXRs. These receptors heterodimerize with several nuclear receptors, such as vitamin D receptor (VDR), thyroid hormone receptor (THR) and peroxisome proliferation activated receptor (PPAR) (*Niederreither* et *Dollé – 2008*).

Retinoid receptors exert their function in the nuclei as transcription factors by binding to retinoid acid response elements (RARE). In the absence of ligand, target gene promoters are silenced by histone deacetylation and chromatin compaction. This results from the recruitment of complexes that contains histone deacetylase (HDAC) coupled with co-repressors (nuclear receptor co-repressor (NCoR) or silencing mediator for retinoid and thyroid hormone receptor (SMRT) and RAR-RXR dimer resulting in histone (*Bourget* et al – *2000*). Retinoids are transported to the nuclei by binding to the cellular retinoid acid binding protein (CRABP). Upon ligand binding to RAR it destabilizes its binding to the co-repressor leading to a destabilization of the ligand binding domain and creating an interface for co-activators. Co-activator complexes activate the transcription machinery by decondensing the chromatin and increase transcription frequency and initiation. These complexes can be: Histone acetyltransferase (HATs), thyroid hormone receptor-associated proteins (TRAP), VDR-interacting protein (DRIP) and Srb and mediator protein-containing complex (SMCC) (*Alltucci* et *Gronemyer – 2001; Bastien* et *Rochette-Egly – 2004*) (Figure 8).

## 2.3 Retinoids as differentiation agents

Retinoids play a preponderant role in embryonic development, as vitamin A deficient diet in mice cause a severe neonatal disorder that affect several organs
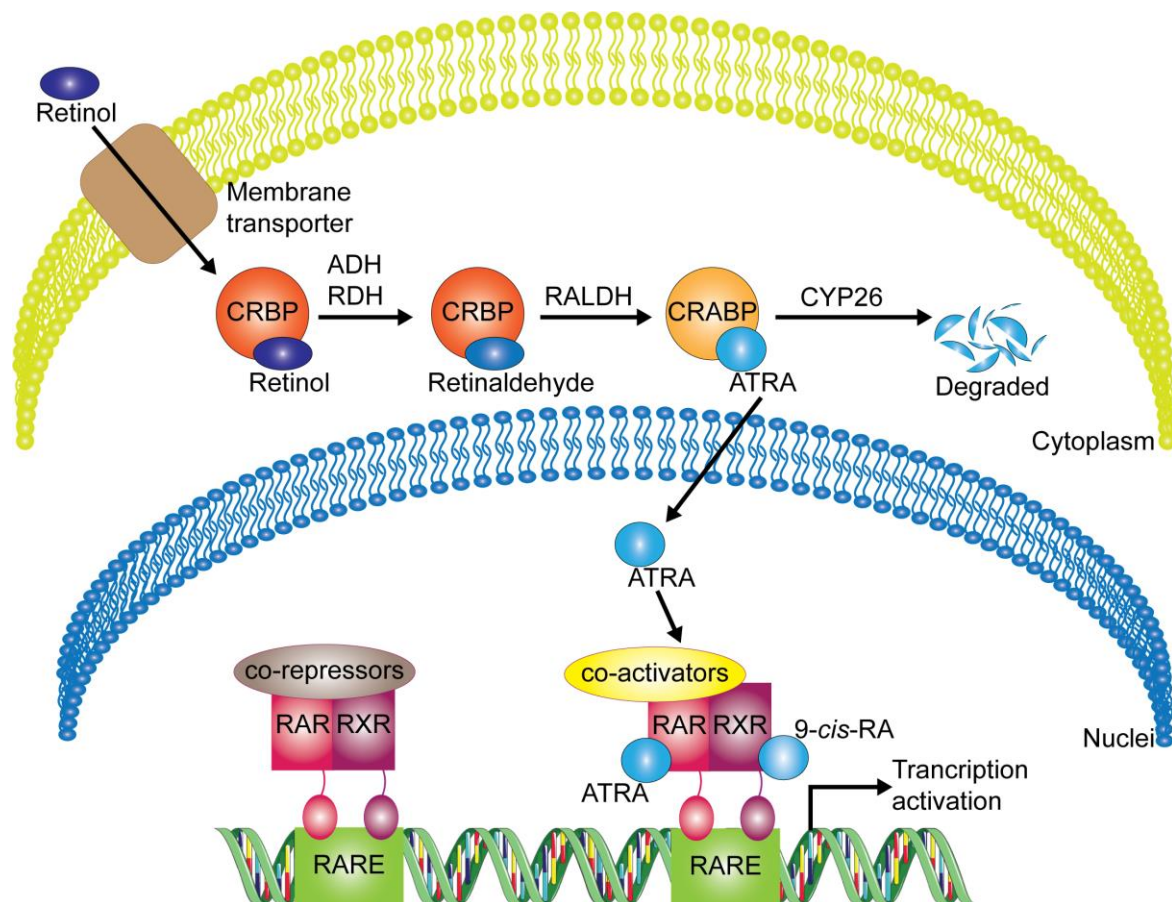


**Figure 8 – Retinoids intracellular processing and transcription regulation.** Uptake of retinol is done by membrane transporters and binds to cellular retinol binding protein (CRBP) in the cytosol. Hydrolysis of retinol into retilnaldehyde occurs by the action of alcohol dehydrogenase (ADH) or retinol dehydrogenase (RDH). This compound is catabolized to the active form ATRA by retinaldehyde dehydrogenase (RALDH). ATRA can then be transported into the nuclei where it binds to retinoic acid receptor (RAR). This receptor might be heterodimerized with retinol X receptor (RXR) and co-repressors bound to retinoid response elements (RARE) and gene promoters, resulting gene transcription inhibition. When retinoids bind to RAR and RXR there is the unbinding of co-repressors and the mobilization of co-activators leading to transcription activation. Additionally, retinoids can be degraded by the action of cytochrome p450 26 (CYP26) enzymes (adapted from *Green* et al – *2016*)

(*Wilson* et al – *1953*). Technological advances allowed for a better understanding of the role of retinoids in development, by using gain of function and loss of function retinoid receptors transgenic animal models. They showed that retinoids regulate embryonic anterior-anterior posterior pattern via regulation of homeobox genes.

Furthermore, they are important for patterning of neural tube and differentiation of motor neurons from neural tube progenitors (*Maden – 2006; Maden – 2007*). Retinoids are also important for the development of inner ear, eye and facial region and forebrain as well as for the differentiation of the heart, lung, kidney and pancreas (*Niederreither* et *Dollé – 2008*).

During mammal embryonic development the major source of retinoids is the maternal retinol, which is taken up by embryonic retinol binding protein 4 (RBP4). It reaches embryonic cells by the action of receptor protein stimulated by retinoic acid 6 (STRA6) where it is transformed to retinaldehyde by retinol dehydrogenase 10 (RDH10). Further cleavage by retinaldehyde dehydrogenases (RALDH) forms different isoforms of retinoids which will act as described in section 4.2 (*Niederreither* et *Dollé – 2008*).

Along embryogenesis retinoids are important for the development of the nervous system, where they regulate patterning and cell differentiation. Together with WNT signalling molecules and fibroblast growth factors, retinoids organize posterior hindbrain and anterior spinal cord formation (detailed reviewed in *Maden – 2007* and *Niederreither* et *Dollé – 2008*).

Retinoids regulate number of primary neurons and differentiation of motor neurons. Primary neurons are the first ones formed and they develop in three parallel rows close to the midline and each row differentiates in motor neurons, interneuron and dorsal sensory neurons. Activation of retinoid signalling increases the number of primary neurons by increasing the neurogenesis inducers X-ngnr-1, Gli3, X-MyT1 and inhibits Zinc2. In addition, retinoids and sonic hedgehog (SHH) work together in the regulation of the number of primary neurons, as SHH overexpression inhibits primary neuron production and this transcription factor is inhibited by retinoids (*Maden – 2007*). Absence of retinoids during development of chick spinal cord reduce the number of islet 1- positive motor neurons and those present had smaller neurites (*Wilson* et al – *2004*). In addition, retinoids were found to be important in the specification of motor neurons subtypes (*Ji* et al – *2006*).

The neurodifferentiation potential of retinoids was studied using both NB cell lines and embryonal carcinoma cells. As retinoids and its receptors work as transcription factors, they were found to regulate the expression of genes encoding signalling proteins, cell surface receptors and other transcription factors. Some of the genes that were found to be activated by retinoids during neurodifferentiation include

nuclear factor κB (NFκB), neurogenin 1, protein kinase Cε (PKCε), ceramide and components of the WNT signalling pathways. While repression of genes that encoded the tyrosine kinase SHP-1 and the WNT inhibitor dickkopf homologue 1 (DKK1) by retinoids was necessary for neurodifferentiation (*Maden – 2007; Mizuno et al – 1997; Verani et al – 2007*).

## 2.4 Retinoids in the treatment of NB

The differentiation role of retinoids made it an interesting drug for cancer treatment and in the 90s they became part of the standard treatment for acute myeloid leukemia and NB.

In vitro studies using NB cell lines, showed that ATRA treatment inhibited cell proliferation and reduced MYCN mRNA expression (*Sidell et al – 1983, Thiele et al – 1985*). A long exposure to ATRA inhibited cell proliferation up to 60 days after treatment initiation. This effect was accompanied with a reduction of the protein levels of MYCN (*Reynolds et al – 1991*). Short treatment of several NB cell lines with ATRA induced growth of neurites and expression of metalloproteinases (MMPs). Moreover, inhibition of MMPs prevented RA response (*Joshi et al – 2007*). The isomer 9-cis-RA was found to induce apoptosis in NB cell lines (*Lovat et al – 1997*) and 13-cis-RA inhibited cell proliferation and increased cell adhesion (*Voigt et al – 2000*).

In vivo studies using SH-SY5Y xenograft showed the inhibition of tumour growth by both 13-*cis*-RA and 9-*cis*-RA (*Rothan et al – 2001*). The synthetic retinoid fenretinide was found to induce cytotoxicity in neuroblastoma cell lines and to be highly effective in neuroblastoma in vivo tumour models.

Expression of RARα, RARβ and RARγ was evaluated in 50 NB patients, and it was found that RARβ was associated with good prognosis. In addition, it was described that activation of this receptor might induced p21-mediated cell cycle arrest (*Cheung et al – 1998*). Recently it was demonstrated that RXRα and RXRβ play opposite roles in neuroblastoma differentiation. While the first contributes to cell cycle arrest and expression of differentiation genes, the latter inhibits these processes (*Girardi et al – 2019*).

Clinical trials using 13-*cis*-RA as treatment for NB patients were stared in the 90s, and in phase II American and European clinical trials it was described that low continuous dose did not affect the survival of the patients (*Finklestein et al – 1992*).

Some concerns were raised about drug efficacy and clinical trial design, and later, a phase I pharmacokinetics clinical trial determining maximal tolerated dosage (MTD) of 13-*cis*-RA was published. This studied allowed to determine that MTD given for 2 weeks/daily was well tolerated with mild side effects (such as dryness of skin and mucus membranes, conjunctivitis and hypertriglyceridemia) and plasma concentrations were effective in in vitro studies (*Villablanca* et al – *1995; Kahn* et al – *1995*). This treatment schedule increased patient survival in high-risk NB patients after intensive chemotherapy, radiotherapy and bone marrow transplantation, and it is part of NB treatment schedule since them (*Matthay* et al – *1999*). A ten-year follow-up study showed that patients treated with 13-cis-RA had better 5-year EFS (*Matthay* et al – *2009*). In addition, efficacy of anti-GD2 treatment was evaluated in combination with 13-cis-RA, IL-2 and GM-CSF showing that the combination of these four molecules significantly improved NB patient survival when compared to the standard care, consisting of the single administration of 13-*cis*-RA (*Yu* et al – *2010*).

### 2.4.1 Resistance mechanisms

A large percentage of patients that respond to 13-cis-RA maintaining low or undetectable minimal residual disease (MRD) levels. However, there are a large percentage of patients that do not respond to this drug. Thus, understanding the mechanisms behind this allows for the development of new therapeutical strategies. A number of potential mechanisms of resistance to this therapy have been proposed.

Alterations in retinoid metabolism might be one of the causes for resistance, and in NB it was described that expression of ALDH1A2 is associated with poor prognosis and resistance to 13-*cis*-RA. This gene codes for a less active ALDH1 isoform, which is responsible for the intracellular oxidation of retinol to retinoic acid (*Hartomo* et al – *2005*).

Studies conducted using retinoid resistant and responsive NB cell lines, allowed for the discovery of different retinoid resistance biomarkers. PBX1, a nuclear protein that forms transcription complexes together with HOX and MEIS, was found to be upregulated in NB cell lines in response to 13-*cis*-RA. This effect was only observed in retinoid sensitive NB cell lines. In NB patients, high expression of PBX1 was associated with benign ganglioneuromas (*Shah* et al – *2014*). Proteins of the homeobox (HOX) family are regulators of cell morphogenesis and play an important

part in neurodifferentiation. Promoter region of HOXC9 was found to be silenced in retinoid resistant NB cell lines and in a constitutive active state in retinoid sensitive NB cells. Protein levels of this transcription factor increased during ATRA induced differentiation (*Mao* et al – *2011*).

Neurofibromin 1 (NF1), a RAS antagonist, plays an important part in cAMP/PKA pathway. This protein was found to influence retinoic acid-induced differentiation of NB cells via the RAS-MEK signalling pathway (*Hözel* et al – *2010*). Knockdown of NF1 in SH-SY5Y maintained their proliferation and colony formation capacity even in the presence of retinoic acid. In the same study, high NF1 expression of NF1 was correlated with better progression free survival in three different patient cohort (*Hözel* et al – *2010*).

# 3    Calcium-sensing receptor (CaSR)

The calcium-sensing receptor is a family C G protein-coupled receptor (GPCR), that was first cloned from bovine parathyroid glands by Edward Brown and colleagues in 1993 (*Brown* et al – *1993*).  Their study described the existence of a bovine parathyroid calcium sensor, that when expressed in *Xenopus laevis* oocytes produced an inward $Cl^-$ current in response to $Ca^{2+}$, $Mg^{2+}$ and $Gd^{3+}$. Additionally, they proposed that the structure of this calcium-sensing receptor has a large extracellular domain (ECD), with 7 transmembrane domains (characteristic of the G protein-coupled receptors), and a large intracellular domain (ICD), as well as extracellular glycosylation sites (*Brown* et al-*1993*). CaSR is mainly expressed in the parathyroid glands, and it is responsible for maintenance of calcium homeostasis. High plasma $Ca^{2+}$ concentrations activate CaSR and lead to a suppression of PTH secretion, reduction of $Ca^{2+}$ excretion by the kidneys and $Ca^{2+}$ deposition in the bones (*Brennan* et al – *2013*). Since its discovery, many studies around the world have shown that this receptor is also expressed in different tissues, such as the intestine, lungs, brain, pancreas, cardiac and immune system where it was shown to plays diverse roles in cellular processes like proliferation, differentiation, gene expression and apoptosis (*Brennan* et al – *2013*). In addition, aberrant expression and function of the CaSR has been found to be associated with calciotropic diseases (that is, dysfunctional extracellular $Ca^{2+}$ homeostasis), but also with the onset of non-calciotropic diseases such as asthma, Alzheimer's, cardiovascular diseases, diabetes and different types of cancer (*Hannan* et al – *2018*). In this chapter, it will be addressed how CaSR expression is regulated, which cellular pathways are activated by this receptor and how its activity is modulated by allosteric modulators and what is the role of CaSR in cancer and particularly in neuroblastoma (NB).

## 3.1    CaSR gene and regulation of expression

In the human genome, the *CaSR* gene is located at chromosome 3q13.3-21 and it lays between the T cell antigen *CD86* (upstream) and cysteine protease inhibitor *CSTA* (downstream) (*Janicic* et al – *1995*). CaSR gene is composed of eight exons, both promoters are in exon 1 while exons 2 to 7 encode the 1078 amino acids peptide that compose the CaSR protein (*Aida* et al – *1995; Garrett* et al – *1995, Brown* et al – *1993*). This gene has two alternative 5'- untranslated regions (UTR) in exons 1A and 1B that shows the presence of two different promoters (P1

and P2). The CaSR gene is highly conserved between species, there is 85% homology between mouse and rat CaSR and about 40% between rodent and human CaSR (*Janicic* et al – *1995*).
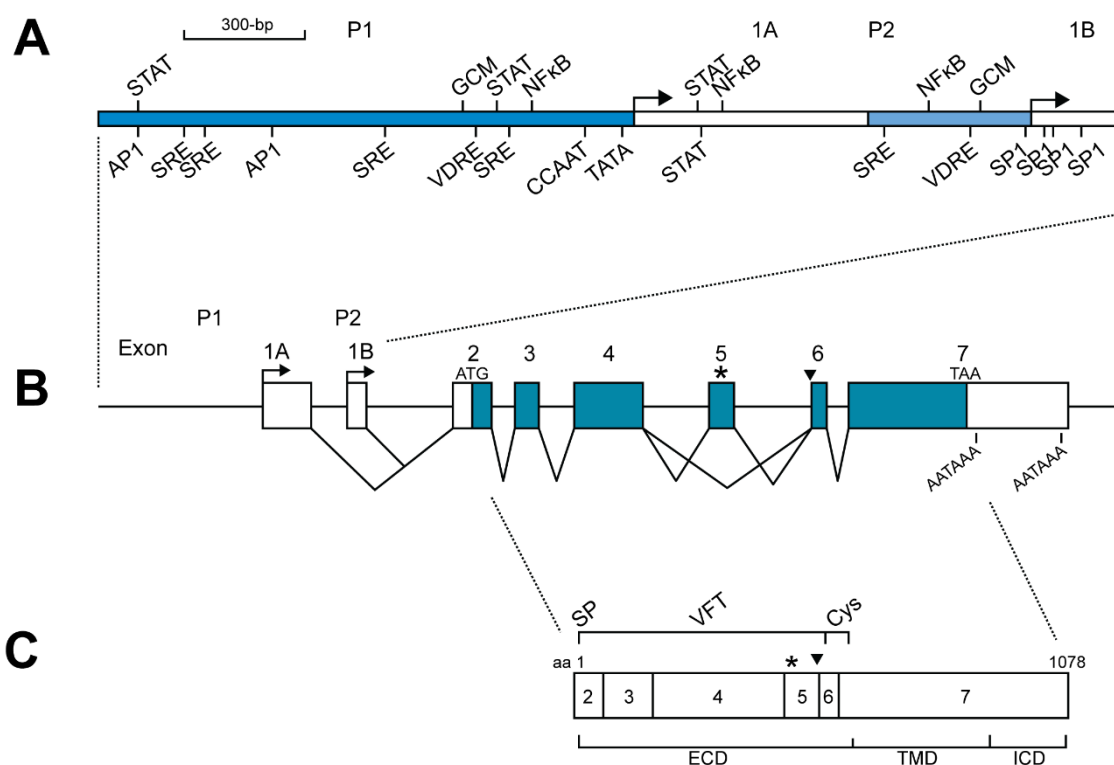


**Figure 9 – Schematic representation of CaSR**. **A –** CaSR gene promoters, promoter P1 and P2 are represented in blue, white bar represents exon 1A and 1B, and transcription of this exons is driven by CCAAT and TATA boxes as well as SP1 sites. signal transducer and activator of transcription (STAT); activator protein 1 (AP1); serum response elements (SRE); vitamin D response elements (VDRE); glial cells missing (GCM); nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). **B –** CaSR gene intron and exon scheme, blue areas represent the translated sequences while white areas correspond to untranslated mRNA sequences. ATG is the start codon, while TAA is the stop codon. Arrowhead indicates an alternative isoform with additional 10 amino acids codded by an alternative mRNA transcript with 50 extra base pairs. Asterix indicates the CaSR isoform lacking exon 5 (77 amino acids less). **C –** CaSR protein composed of 1078 amino acids and coded by exon 2-7 of CaSR gene. Signal peptide (SP); venus fly trap domain (VFT); cysteine rich domain (Cys); extra cellular domain (ECD); transmembrane domain (TMD); intracellular domain (ICD) (adapted from *Hendy* et *Canaff – 2016*)

Human CaSR has two promoter regions (P1 and P2), which result in different 5'UTR and different lengths of CaSR transcripts in human parathyroid glands (5.4kb, 4.8kb,10kb, 4.2kb) (*Garret* el at – *1995; Chikatsu* et al – *2000*). In addition, transcripts lacking exon 3 or exon 5 have been found in different tissues. Deletion of exon 3 generates a protein exhibiting low expression, which is unable to traffic to the

cell surface, and is present in thyroid TT cells (*Freichel* et al – *1996*), placenta (*Bradbury* et al – *1998*) and parathyroids, thyroid and kidney (*D'Souza-Li* et al – *2001*). A CaSR transcript lacking exon 5 was found expressed in keratynocytes and leads to a CaSR protein lacking 77 amino acids in the extracellular domain. This variant has a lower response to $Ca^{2+}$ and a dominant negative effect in the full-length CaSR, and in addition the ratio of full-length to truncated protein varies during keratinocyte differentiation (*Oda* et al – *1998*).

The two CaSR promoters have different starting regions, P1 has a TATA box at -26 and CCAAT box at -110 relative to start site and P2 has a Sp1/3 start site. In addition, both promoters have different binding sites for transcription factors (*Hendy* et *Canaff* – *2016*). These features might explain the different CaSR expression patterns between tissues and understanding how CaSR expression is regulated might allow for a better treatment of diseases where CaSR levels are aberrant. Regulation of gene expression can happen during transcription, by the binding of different transcription factors to the transcription regulation sites located around the promoter region of the gene. Around the two CaSR promoters, different putative regulatory elements have been identified (*Hendy* et *Canaff* – *2016*) (Figure 9).

Validation of these putative sites was achieved using substances which affect the activity of transcription factors that bind to different response elements located in the CaSR promoter. Both CaSR promoters have one vitamin D response element (VDRE), and it has been demonstrated by several groups that vitamin D increases the mRNA expression of *CaSR* in kidney and in parathyroid cells (*Yao* et al – *2005*). In addition, treatment of colon cancer cells lines with the active vitamin D compound calcitriol increases CaSR expression and inhibits their proliferation (*Fetahu* et al – *2014*). Expression and activity of transcription factors STAT and NFκB is increased by proinflammatory cytokines. Two cytokines, IL6 and IL1β, increased CaSR mRNA and protein expression in parathyroid, thyroid and kidney when injected into rats. Additionally, it was demonstrated that IL1β and TNFα increase CaSR expression due to their activation of the transcription factor NFκB (*Canaff* et *Hendy* – *2005*), while IL-6 exerts its functions via STAT1/3 and Sp1/3 transcription factors (*Canaff* et al – *2008*). In a recent review, the authors noticed that IL-6 induces a more rapid decrease of PTH than IL1β, and they hypothesised that IL-6 might induce a more rapid mRNA transcription and protein synthesis via eukaryotic initiation factor 4F (*Hendy et Canaff* – *2016*). The effect of cytokines in the

expression of CaSR was also studied in cell types where CaSR has low expression levels. Fetahu and her colleagues have shown that CaSR mRNA and protein levels increased when colonocytes were treated with TNFα, IL1β and IL6 (*Fetahu* et al – *2014*). Murine macrophages stimulated with lipopolysaccharide released TNFα, that up-regulated CaSR that worked as a negative regulator of the secretion of this cytokine (*Kelly* et al – *2011*).

### 3.2   GPCR and CaSR signalling

GPCRs represent the largest class of membrane-bound proteins in the human genome, they are a very diverse protein family, with a high range of physiological functions and ligands (*Hauser* et al – *2017*). In spite of this diversity, all GPCR families have the same structure: N-terminal extracellular domain (ECD); a seven transmembrane α-helices connected by intracellular and extracellular loops (7TM) and a C-terminal transmembrane domain (CMD). GPCRs were first classified into six different families based on gene homology: class A (rhodopsin-like), class B (secretin-like), class C (metabotropic glutamate), class D (fungal mating pheromone receptors), class E (Cyclic AMP receptors), class F (frizzeled/smoothened) (*Kolakowski Jr LF – 1994*). Ten years later Fredriksson and colleagues developed the GRAFS systems, where they grouped more than 800 GPCRS in five different families using phylogenetic methods. Those families are called: glutamate-like (class C), rhodopsin like (class A), adhesion, frizzle and secretin like (class B). (Fredriksson et al-2003), which is the most accept classification. (*Alexandre* et al – *2018*).

Differences between the families of GPCRs are mainly in their extracellular domain and the way they bind their ligands. Secretin-like family GPCRs (class B) contain cysteine bridges in their N-terminal, important for ligand binding, their main ligands are large peptides. Adhesion-like GPCRs are transmembrane GPCRs with adhesion-like motifs in their N-terminal region, which vary in size and are rich in glycosylation sites and proline residues. Frizzle family includes the taste receptors, that have a short N-terminal sequence and are not predicted to have ligand-binding sites. Furthermore frizzle GPCRs have a 200 amino acid N-terminal sequence and participate in the binding of Wnt motifs, these two types of receptors have consensus sequences in the TM loop 2, 5 and 7. In rhodopsin-like family (class A), the ligand binding is done in the cavity of the 7TM, and all of the receptors belonging to this family contain a NSxxNPxxY motif in TM7 and a DRY motif or D(E)-R-Y(F) sequence

at the border between the TM7 and the IL2. The glutamate family (class C) has a very large N-terminal ECL, with two lobes that close around the ligand and form a
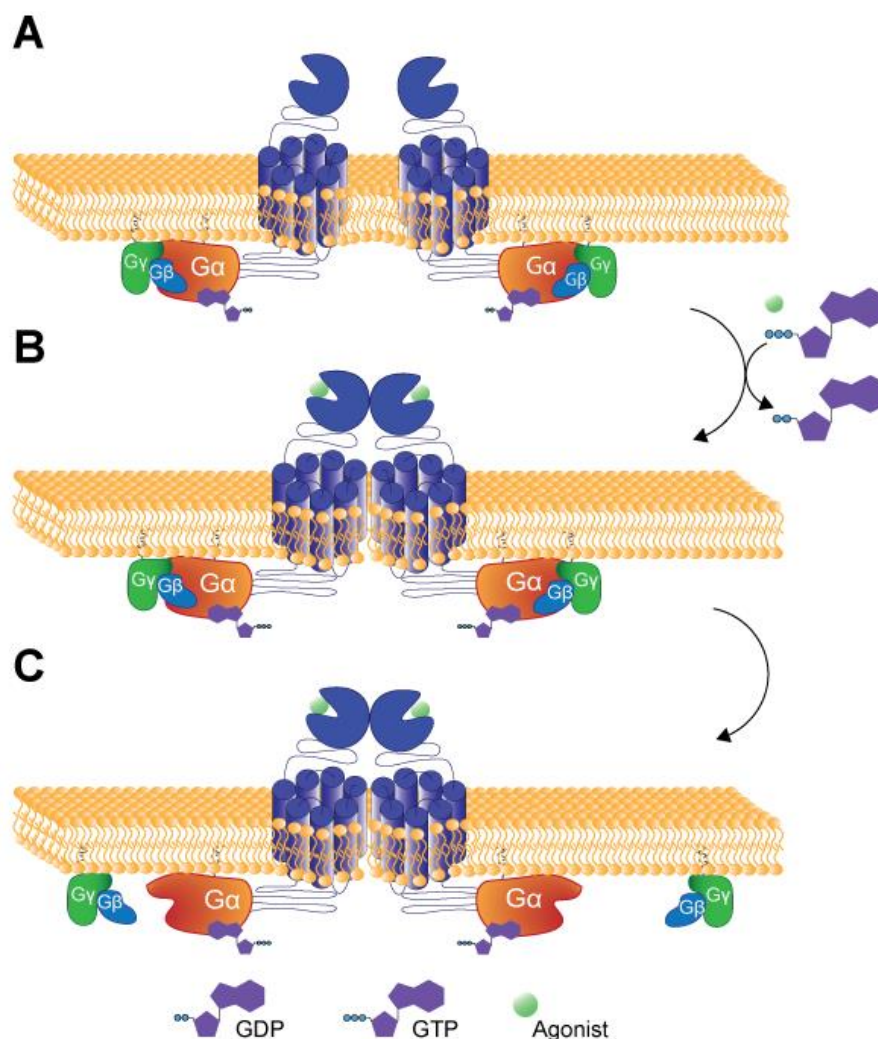


**Figure 10 – Class C GPCR activation**. Family C GPCRs are present in the cells membrane as monomers and are associated to the GDP-bound trimeric Gαβγ **(A)** GPCR ligand binds to the opened VFT that closes and gets closer to both the 7TM and to the closest monomer, forming a dimer. At the same time there is a GDP to GTP transfer in the Gα subunit by the guanine exchange factor **(B)**. This exchange activated the trimeric G-protein, this results in a dissociation of the Gα subunit from the Gβγ dimer **(C)**.

"Venus fly trap" (VFT) domain (*Fredriksson* et al – *2003*).

CaSR is a class C GPCR and its ECD crystal structure was recently published (*Zhang* et al – *2016*, *Geng* et al – *2017*). Geng and colleagues obtained the crystal structure both in the inactive and active form, describing new binding sites for $Ca^{2+}$, $PO_4^{3-}$ and L-Trp. Furthermore, they demonstrated that amino acids and $Ca^{2+}$ function may represent co-agonists of CaSR. Ligand binding to CaSR is similar to other class C GPCRs, it induces the closure of the VFT, forming a homodimer

interface between the large lobule of VFT and the Cystein rich domain of ECD. After ligand binding there is a reduction of the distance between both ECDs, that might result in rearrangements of the 7TM and the activation of G proteins attached to the receptor (*Geng* et al – *2017*) (Figure 10).

In its inactive form, CaSR monomers are in proximity, each bound to their respective heterodimeric G protein. This heterodimer is composed of three subunits, Gα, Gβ and Gγ bound to a GDP molecule and are the main players in the intracellular signalling cascade of GPCRs (*Rosenbaum et al – 2009*). Upon binding of the ligand VFT, there is a closure of this domain and a conformational change that reduces the distance between the two monomers. In addition, there is a release from the GDP bound to the G protein heterodimer and coupling of a GTP to the G-protein. This exchange leads to a disassembly of the Gβ/γ subunits from the Gα, activating
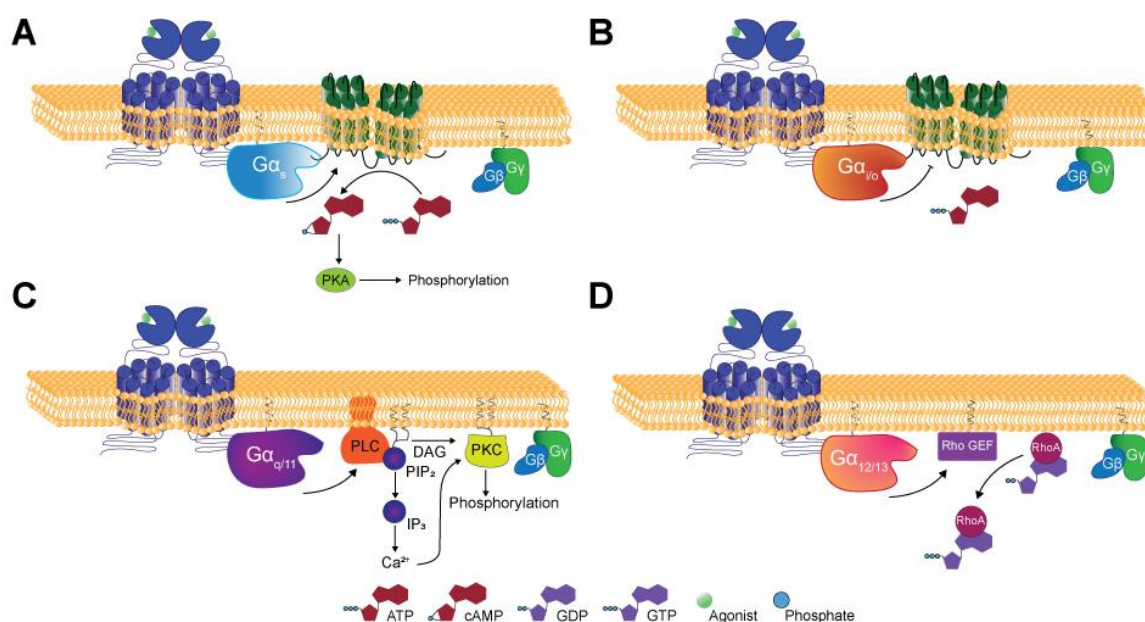


**Figure 11 – GPCR signal transduction**. GPCR signalling is dependent on the family of the Gα subunit. Signalling though Gα$_s$ activates adenyl cyclase (AC) inducing the formation of cAMP from ATP, this will in turn activate PKA and the phosphorylation of several downstream proteins **(A)**, activation of Gα$_i$ will have the opposite effect inhibiting the activity of AC **(B)**. Activation of the Gα$_s$ activates PLC, leading to the cleavage of PIP$_2$ to generate DAG, which activated PKC, and IP$_3$, which releases Ca$^{2+}$ **(C)**. In the case of activation of Gα$_{12/13}$ there is an activation of RhoGEFs leading to an exchange of GDP to GTP bound to RhoA **(D)**.

different signalling cascades depending on the Gα subunit present (*Conigrave* et *Ward – 2013*) (Figure 11). There are four main families of Gα subunits that induce the activation of different intracellular signalling cascades: Gα$_s$, Gα$_{i/o}$, Gα$_{q/11}$ and

Gα$_{12/13}$ (*Simon* et al – *1991*). Gα$_s$ and Gα$_{i/o}$ affect the activity of adenylcyclases (ACs), with Gα$_s$ activating ACs leading to the cleavage of adenosine 5'-triphosphate (ATP) to generate 3'-5'-cyclin adenosine monophosphate (cAMP), whereas Gα$_{i/o}$ inhibits this reaction (*Hurley* – *1999*). The resulting cAMP then binds to and activates protein kinase A (PKA) leading to the phosphorylation of different downstream effector proteins, such as the transcription factor cAMP response element binding (CREB) (Figure 11A-B). Another Gα family, Gα$_{q/11}$ activates β isoforms of phospholipase c (PLC), this will lead to the hydrolysis and conversion of phosphatidylinositol 4,5-biphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$), the latter then inducing release of Ca$^{2+}$ release from endoplasmic reticulum (ER) stores. Protein kinase C (PKC) is activated by an increase of intracellular Ca$^{2+}$ and DAG and will further activate several downstream signalling pathways such as c-Jun N-terminal kinase (JNK) and p53 (*Syrovatkina* et al – *2016*) (Figure 11C). Although there is limited knowledge of how Gα$_{12/13}$ does its effector functions, it has been demonstrated that this G-protein activates Rho guanine nucleotide exchange factors (RhoGEFs) leading to a GDP to GTP exchange in the RhoA GTPase, inducing not only a remodelling of the actin fibres but also activation of different transcription factors, like p38 and JNK (*Syrovatkina* et al – *2016*) (Figure 11D). In addition to G-protein dependent signalling, GPCRs also signal through β-arrestins, which have been shown to be adaptors of agonist-induced endocytosis and ubiquitination (reviewed in *Jean-Charles* et al – *2017*).

CaSR signals mainly though Gα$_{i/o}$, Gα$_{q/11}$ and Gα$_{12/13}$, in some cell types CaSR activates G$_s$ (reviewed in *Chavez-Abiega* et al – *2020*). In HEK293 cells, CaSR is constitutively internalised via a β-arrestin mediated signalling and in the presence of its main ligand, Ca$^{2+}$, it signals via Gα$_{i/o}$ and Gα$_{q/11}$ (*Thomsen* et al – *2012*, *Mos* et al – *2019*). The CaSR may exhibit "ligand bias", which is the ability of different ligands to stabilize different receptor conformations and activate one G-protein in favour of another (reviewed in *Leach* et al - *2015, Kenakin* et *Christopoulos* – *2013*). Biased signalling is tissue dependent since it can be subject to downstream signalling molecules (PLC, G-proteins, Rho, cAMP) but also on the ligands that are present in tissue (*Conigrave* et *Ward* – *2013*, *Leach* et al – *2015*). Biased signalling of CaSR is seen not only as a response to endogenous molecules but also to synthetic compounds created to modulate the activity of CaSR, usually referred to as "allosteric modulators".

### 3.3 Allosteric modulators of CaSR

Allosteric modulators are molecules that stabilize a particular receptor conformation, increasing or decreasing the affinity of the ligand binding sites (*Bartuzi et al – 2018*). Molecules that activate CaSR by mimicking $Ca^{2+}$ or potentiating its effect are often called calcimimetics. This class of drugs can be classified into type I, orthosteric compounds that single handed activate CaSR (can be inorganic and organic polycations ($Mg^{2+}$, $Gd^{3+}$)), or type II, compounds that need the presence of an orthosteric compound to exert its function (can be synthetic or natural small molecules (cinacalcet, NPS-568, spermine, aromatic amino acids, glutathione)). Compounds that inhibit the activation of CaSR are often called calcylitics and are small organic molecules that act as negative allosteric modulators of CaSR (NPS-2143, ronacaleret) (*Nemeth* et *Goodman – 2016*) (Figure 12).

Calcimimetics and calcylitics have been widely studied for the treatment of several diseases, both calciotropic and non-calciotropic (*Hannan* el al – *2018*), however calcimimetics have been the most successful as clinical treatments. Cinacalcet (CIN) (Mimpara®; Sensipara®) was approved for clinical use in 2004, becoming the first GPCR allosteric modulator to reach the pharmaceutical market (*Bräuner-Osbourne* et al – *2007*) (Figure 12). CIN was approved for treatment of severe hypercalcemia in patients with parathyroid carcinoma or primary hyperparathyroidism who cannot have a parathyroidectomy as well as chronic kidney disease patients with secondary hyperparathyroidism (SHPT) (*Barman Balfour* et *Scott – 2005*). *In vitro* studies demonstrated that CIN increases intracellular $Ca^{2+}$ levels in HEK-CaSR, in addition this calcimimetic inhibited the secretion of PTH in bovine parathyroid cells and increases the secretion of calcitonin by MTC 6-23 cells (a medullary thyroid carcinoma cell line) (*Nemeth* et al – *2004*). Oral administration of CIN to healthy rats led to a dose dependent reduction of PTH (maximum response after 1-hour) and ionised $Ca^{2+}$ (lowest peak seen after 4-hours), there was also an increase of calcitonin release (highest after 2-hours). CIN is more stable than its predecessor NPS R-568, a calcimimetic that failed during clinical trials due to its pharmacokinetic and metabolic properties (*Nemeth* et *Goodman – 2016*).

Use of CIN decreases patient mortality and the occurrence of cardiovascular events in haemodialysis patients, however it has been reported that gastrointestinal side effects (such as nausea and vomiting) reduce patient compliance and drug dosage (*Komaba* et al – *2015, Gincherman* et al – *2010*). This has created a need to develop calcimimetics with higher tissue specificity. In recent years an intravenous calcimimetic, etelcalcitide (AMG 416), was approved to for the treatment of SHPT in
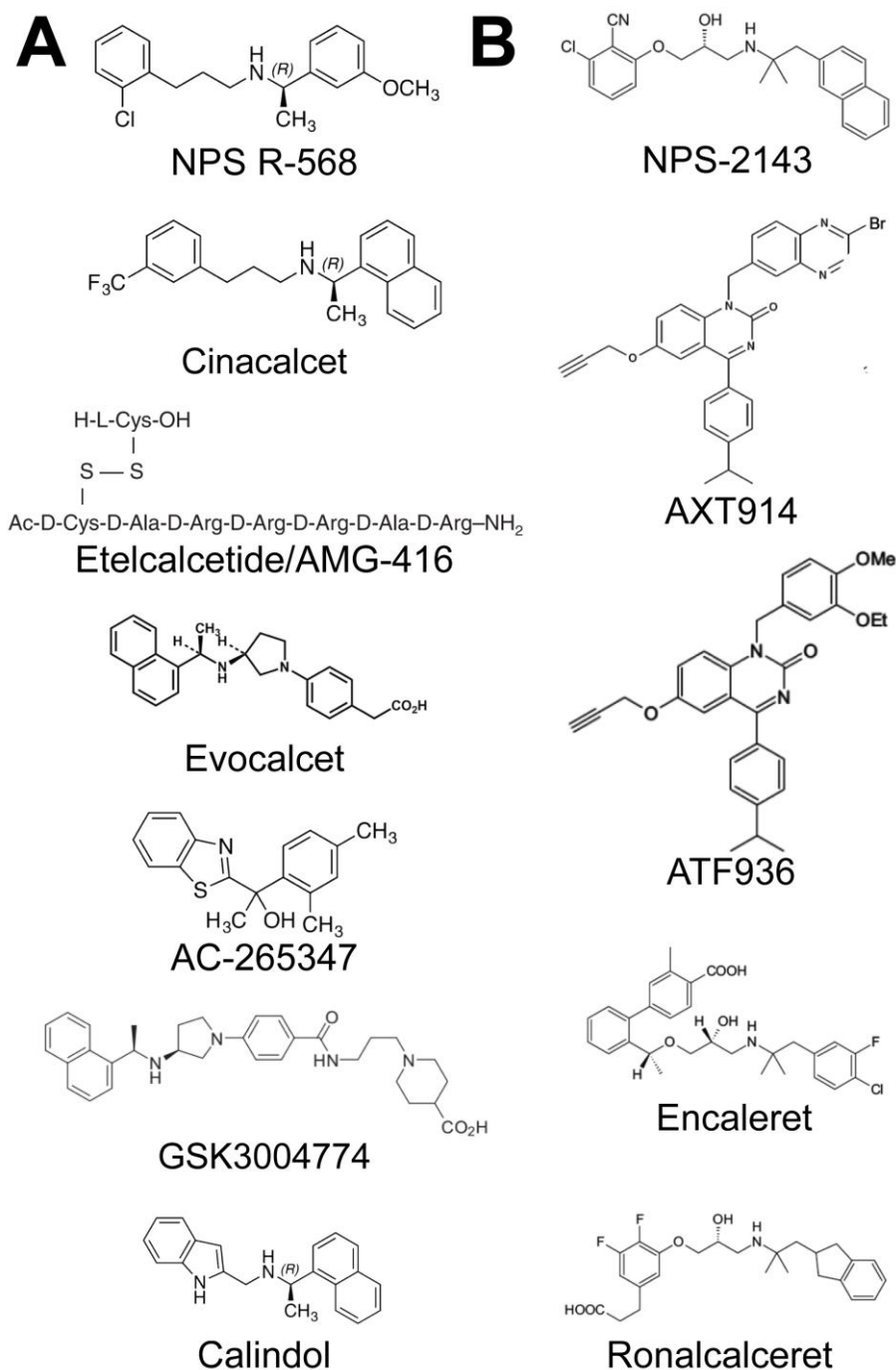


**Figure 12 – Chemical structure of CaSR allosteric modulators**. Structures of calcimimetics **(A)** and calcylitics (B) (adapted from *Nemeth* et *Goodman – 2016*).

chronic kidney patients undergoing dialysis (*Patel* et *Bridgeman – 2018*). Unlike CIN, which binds to the CaSR transmembrane domain, etelcalcitide binds to the extracellular domain acting as an agonist (*Alexandre* et al – *2015*) (Figure 12). In a clinical trial comparing the effectiveness of CIN versus etelcalcitide, the later was found to be as effective as CIN in reducing PTH concentration in patients undergoing haemodialysis with moderate to severe SHPT. However, it was seen that etelcalcitide induced nausea and vomiting to the same extent as CIN (*Block* et al – *2017*). In an attempt to reduce the secondary effects of CIN and increase its bioavailability, Japanese researchers have developed evocalcet. In preclinical studies, authors found that evocalcet significantly reduced serum PTH at a dosage of more than 0.03mg/kg while a dosage of 1mg/kg of CIN had a similar effect. In addition, it was found that evocalcet caused less gastric disturbances than CIN (*Kawata* et al – *2018*). This preclinical data was further supported by human clinical trials, where evocalcet was shown to be non-inferior to CIN and to cause less gastro-intestinal adverse effects (*Fukagawa* et al – *2018*, reviewed in *Akizawa* et al – *2019*). Evocalcet has recently been approved in Japan for the treatment of patients undergoing peritoneal dialysis or haemodialysis with SHPT (*Akizawa* et al – *2019*).

Calcilytics were originally developed as an oral alternative treatment for osteoporosis, a current therapy including the systemic administration of abaloparatide, an analog of PTHrP that targets PTH1R and increases bone mass density reducing risk of fracture (*Clynes* et al – *2020; Leder* et al – *2015*). Although there have been three calcilytics that have reached clinical trials for treatment of postmenopausal women with osteoporosis (ronacalceret, encaleret/JTT-305/MK-5442 and AXT914) none of them passed phase 2. All had similar pharmacodynamics and pharmacokinetics, causing dose-dependent and rapid increase of PTH levels. Ronacaleret is the most studied calcilytic for the treatment of osteoporosis in post-menopausal women. Clinical studies comparing the effect of ronacalceret to terapatatide (PTH 1-34) and alendronate and concluded that this calcilytic had a mild effect in bone mass density and that there was a lack of anabolic window in bone turnover markers. In addition, ronacalceret affected renal CaSR leading to an increase of plasma $Ca^{2+}$ levels, tipping the risk-benefit scale of this calcilytic (*Fitzpatrick* et al – *2011a*, *Fitzpatrick* et al – *2011b*). Clinical trials evaluating the effect of other calcilytics had the same outcome. AXT914 increased bone turn over markers, however efficacy was lower than the available standard treatment, a daily

dose of teriparatide. It also induced a dose-dependent increase of $Ca^{2+}$, leading to safety concerns and to a halt in the development of AXT914 (*John* et al – *2014*). Encaleret was given daily to oesteopenic postmenopausal women in a randomized, double-blinded, placebo-controlled clinical trial. After the first month of treatment, women receiving encaleret had a significant reduction of bone turn-over markers, however after 6 months of treatment these parameters were significantly higher than the placebo treated group. Observed changes in the encaleret treated group were still lower than the standard of care and there were reports of hypercalcemic events, both contributed to a halting of further development of this drug (*Halse* et al – *2014*) (reviewed in *Nemeth* et *Goodman* – *2016*).

CaSR plays a plethora of roles in a great number of tissues and diseases, for this reason there has been an attempt to repurpose CaSR modulators for other diseases (*Hannan* et al – *2019*). Preclinical studies have been successful in demonstrating the effectiveness of nebulised calcilytic for the treatment of asthma, demonstrating a new way to use these CaSR antagonists (*Yarova* et al – *2015*). Study of allosteric modulators of CaSR has been evolving with the aim of finding tissue specific compounds that allow for lower impact on plasma $Ca^{2+}$ level and the adverse effect that come from it. This premise allowed for the development of study of several calcimimetics that induce a CaSR bias signalling different from CIN: Etelcalcetide, evocalcet, AC-265347, BTU-compound-13, GSK3004774, calindol. Although two of these calcimimetics have been approved for clinical use, the other calcimimetics have properties that make them interesting tools to study CaSR biased signalling (*Leach* et al – *2015*). An attempt to generate a gastro-intestinal specific calcimimetic lead to the development of GSK3004774. This calcimimetic was shown to have low intestinal permeability, leading to a low systemic concentration and high luminal stability and fecal recovery (*Sparks* et al – *2017*). Recently, GSK3004774 and CIN were shown to have a different impact in an acute mouse model of colitis (*Elajnaf* et al – *2019*). Another calcimimetic that was found to have a differential effect than CIN was AC-265347, as it was first described to have a lower impact on calcitonin secretion by thyroidal C-cells than CIN. In addition, mutation studies have shown that AC-265347 binds to a different place than CIN in the 7TM of CaSR (*Ma* et al – *2011*). This calcimimetic has been widely studied by the group of Katie Leach, they reported a bias of AC-265347 towards pERK1/2 phosphorylation versus $Ca^{2+}$ mobilization (*Leach* et al – *2016*). In addition, AC-265347 was described to have a

higher potency than CIN in IP$_1$ accumulation assay, and to have agonist properties, since it induced Ca$^{2+}$ mobilization and IP$_1$ accumulation in the absence of Ca$^{2+}$(*Cook* et al – *2015*). In a recent study, AC-265347 was found to have a bias towards Rho activation versus Ca$^{2+}$ mobilization in the presence of both Ca$^{2+}$ and Sr$^{2+}$ (*Diepenhorst* et al – *2018*).

### 3.4  Dual role of CaSR in cancer

CaSR was demonstrated to play a great number of functions in different tissues, in tumorigenesis it plays different roles depending on the tissue where it originates (*Brennan* et al – *2013*). As shown in Table 5, CaSR can work both as an oncogene and tumour suppressor gene in different neoplasias. The mechanisms that CaSR uses to play these divergent roles is not fully understood, however several possible mechanisms have been uncovered.

The role of CaSR in breast cancer has been widely studied, with most groups having used different breast cancer cell lines to understand it. A study from the Wysomerski group used Comma D cells, a not fully transformed murine breast cell line, and primary cultures of mouse mammary epithelial (MMECs) cell lines to study the G-protein coupling of CaSR in normal and malignant breast cells. These two cells respond oppositely to an increase in Ca$^{2+}$ concentration, while stimulated MMECs reduced cAMP and the secretion of PTHrP, stimulated Comma D cells to increase their secretion of PTHrP and have higher levels of cAMP. Using binding studies, the authors demonstrated that this antagonistic effect was due to a G-protein switch from CaSR; in normal breast tissue CaSR binds to Gα$_{i/o}$ whereas in malignant breast cells CaSR binds to Gα$_s$ (*Mamillapalli* et al – *2010*). In other studies, activation of CaSR, using both Ca$^{2+}$ and spermine, increased the proliferation of MCF-7 cells by activation of PLC, phosphorylation of ERK1/2 and metalloproteinases (MMPs) (*El Hiani* et al - *2009a*, *El Hiani* et al - *2009b*). Downregulation of CaSR in estrogen receptor-positive and negative cells, a prognosis predictor in breast cancer, was found to increase the resistance of these cells to paclitaxel, a chemotherapy agent, due to an up-regulation of survivin, an anti-apoptotic agent (*Liu* et al – *2009*). These effects were found to be dependent on the expression of BRCA1, a breast cancer oncogene (*Promkan* et al – *2011*). CaSR activation with Ca$^{2+}$ increases the migration of breast cancer cell lines via pERK and PLCβ (*Saidak* et al – *2009*) as well as the angiogenic potential of these cells, by

secreting granulocyte-macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF) and macrophage derived chemokine (MDC) (*Hernández-Bedolla* et al – *2015*). More recently, it was demonstrated that CaSR plays an important role in the generation and development of bone metastasis. Specifically, overexpression of CaSR in MDA-MB1-23 cells induces more aggressive bone lesions than cells where CaSR activity is inhibited. This results from an increased secretion of epiregulin by MDA-MB1-23-CaSR cells, upregulating the expression of osteoprotegerin, an osteoclastogenesis inhibitor (*Boudout* et al – *2017*). Recently an extensive study using a cohort of 652 breast cancer tumours demonstrated that high expression of CaSR results in a significantly shorter patient survival. In addition, in a transgenic mouse model with a breast-specific deletion of CaSR in breast cancer cells generated smaller tumours and its *ex vivo* stimulation with $Ca^{2+}$ increased AIF mediated apoptosis of cancer cells (*Kim* et al – *2016*).

Study of a large cohort of prostate cancer patients (1241 tumours) showed that CaSR levels were higher in cancer cells than in normal prostate tissue, additionally, tumours with CaSR abundance were more lethal (*Ahearn* et al – *2016*). In transgenic tumour models, CaSR levels increased during disease progression, and high dietary $Ca^{2+}$ accelerated cancer cell proliferation and microinvasion (*Bernichtein* et al - *2016*). It was demonstrated that in prostate cancer cell lines, CaSR signals via $G\alpha_{12}$ and RhoGEF to activate both ChoK and induce filamin A cleavage, poor prognosis markers of prostate cancer (*Huang* et al – *2011*, *Huang* et al – *2017*).

CaSR was also found to have an oncogenic role in gastric cancer, in a small patient cohort (91 patients) its expression was higher in cancer cells than in normal gastric cells. Using gastric cancer cell lines, authors showed that activation of CaSR with $Ca^{2+}$ increased their proliferation and telomerase activity via AKT phosphorylation (*Xie* el al – *2019*). In addition, CaSR activation increased migration and invasion of tumour cells due to an increase of AKT, mTOR and p70 phosphorylation (*Xie* et al – *2017, Zhang* et al – *2019*).

This GPCR is also an important player in the generation of metastasis in renal cell carcinoma, where primary cells from bone metastasis have a higher expression of CaSR and its activation increases the generation of metastasis in mouse tumour models (*Joeckel* et al – *2014, Frees* et al – *2018*). In intrahepatic cholangiocarcinoma (ICC), high CaSR expression is associated with a lower patient

survival and a higher number of metastases. Activation of CaSR increases proliferation, migration and invasion of ICC cell lines (*Fang* et al – *2020*).

Using a small cohort of colon cancer patients (n=54), when considering the

| Neoplasia | | Mechanism | Reference |
|---|---|---|---|
| **Breast cancer** | O | • CaSR expression correlated with poor prognosis features and lower survival<br>• CaSR present in normal tissue signals via $G\alpha_i$ and in cancer cells signals via $G\alpha_s$<br>• Activation of CaSR increases cell line tumorigenic and metastatic capacities | *Mamipalli* et al – *2008*; *Boudout* – *2017*; *Promkar* et al – *2011*; *El Hiani* et al - *2009a*, *El Hiani* et al - *2009b*; *Hernández-Bedolla* et al – *2015* |
| **Pancreatic cancer** | TS | • Low CaSR expression associated with poor survival<br>• CaSR inhibits tumorigenesis via NCX1/$Ca^{2+}$/β-catenin pathway | *Tang* et al – *2016*; *Hummel* et al – *2014* |
| **Prostate cancer** | O | • Cancer cells have higher CaSR levels than non-malignant cells<br>• CaSR signals via Gα12 and RhoEGF to induce Chok and filamin A mediated tumorigenesis | *Ahearn* et al – *2016*; *Benichtein* et al – *2016*; *Huang* et al – *2017*; *Huang* et al – *2017*; |
| **Gastric cancer** | O | • Positive correlation of CaSR and poor prognosis features<br>• Inhibition of CaSR inhibited malignant features of cell lines via Bcl2, caspase 3 and Bax<br>• Activation of CaSR in tumour models increased metastasis | *Xie* et al – *2017*; *Xie* et al – *2019*; *Zhang* et al - *2019*; |
| **Renal carcinoma** | O | • Levels of CaSR were higher in patients with bone metastasis<br>• CaSR overexpression increases metastatic potential in *in vivo* tumour models | *Joeckte* et al – *2014*; *Frees* et al – *2018*; |
| **Colon cancer** | TS | • Overexpression of CaSR decreased proliferation potential of cell lines<br>• CaSR expression increased during cell differentiation<br>• CaSR is inhibited by oncogenic miRs | *Aggarwal* et al – *2016*; *Aggarwal* et al – *2015*; *Fetahu* et al – *2014a*; *Fetahu* et al – *2016*; *Sign* et al – *2013a*; *Sign* et al – *2013b*; *Sign* et al – *2013c*; |
| **Intrahepatic cholangiocarcinoma (Liver)** | O | • CaSR expression associated with more metastatic and aggressive tumours<br>• Activation of CaSR increases tumorigenic potential of cell lines | *Fang* et al – *2020*; |
| **Lung adenocacinoma** | TS | • CaSR expression associated with longer OS | *Wen* et al – *2015* |
| **Neuroblastoma** | TS | • CaSR expression associated good prognosis markers<br>• Overexpression reduced cell lines malignance<br>• CIN inhibits NB tumour growth | *de Torres* et al – *2009*; *Casalà* et al -*2012*; *Rodríguez-Hernandéz* et al – *2016* |

**Table 1** – **Dual role of CaSR in cancer**. O – oncogene, TS – tumour suppressor

incidence of this type of cancer (the third most common), it was shown that CaSR

had a higher expression in normal colon than cancer cells (*Aggarwal* et al – *2016*). Treatment of colon cancer cell lines with $Ca^{2+}$, calcimimetics, cytokines or 1,25-$D_3$ diminished their proliferative rate and induced a more differentiated status (*Aggarwal* et al – *2016, Fetahu* et al – *2016, Singh* et al – *2013a, Singh* et al – *2013c*). To date, most studies have demonstrated a role of CaSR as a tumour suppressor in colon cancer.

Low expression of CaSR was associated with poor prognosis and shorter overall survival (OS) survival for pancreatic cancer patients (n=65). Using cell lines, authors further demonstrated CaSR's tumour suppressive role, its activation using $Ca^{2+}$ and spermine reduced cell proliferation, cologenic and tumorigenic capacity (*Tang* et al – *2016*). In a cohort of 117 lung adenocarcinoma patients, high CaSR expression was associated with a better prognosis and longer OS (*Wen* et al – *2015*).

### 3.5 CaSR in Neuroblastoma

The first paper relating CaSR to NB was published more than ten years ago and demonstrated that high levels of *CaSR* mRNA expression were found in NB tumours with features associated with good prognosis (younger patients; clinical stages 1,2 and 4S; differentiated tumours). In addition, NB tumours that received differentiating treatment and had a more differentiated morphology were positive for CASR, including patients in which tumours had no expression of CaSR. Expression of this GPCR was increased during the process of differentiation and it was hypothesised that CaSR might work as a tumour suppressor gene in NB (*de Torres* et al – *2009*). Polymorphism rs1801725, present in the exon 7 of CaSR and being responsible for a less active receptor (*Yun* et al – *2007*), was found to be correlated with neuroblastic tumours with more aggressive features, stage 4 with undifferentiated histological features (*Masvidal* et al – *2013*). It was demonstrated that CaSR promoters were hypermethylated in NB cell lines and in 25% of NB primary tumours. Tumours expressing CaSR after treatment, showed a lower hypermethylation of P2. In addition, presence of rs7652589 and rs1501899 in NB tumours was correlated with a lower expression of CaSR mRNA, independently of hypermethylation of CaSR P2 (*Masvidal* et al – *2017*). Although there was an association of different CaSR polymorphisms with clinical features none of the observations were predictors of clinical outcome.

Hypermethylation of CaSR promoter was reduced after treatment with demethylation agents leading to CaSR expression. Furthermore, overexpression of CaSR in two NB cell lines reduced their proliferative and their anti-tumorigenic capacity. Apoptosis of these cells was induced by $Ca^{2+}$, which was described to occur via ERK1/2 phosphorylation (*Casalá* et al – *2012*).

After establishing CaSR as a tumour suppressive gene in NB, the group focused on how this molecule can be targeted. In an attempt to repurpose CIN for the treatment for NB, it was shown that *in vitro* CIN induced an ER-mediated apoptosis of NB cell lines and prolonged exposure to this calcimimetics induced cell death and cell differentiation. The most important finding was the in vivo effect that CIN had in NB xenograft models; it inhibited tumour growth and increased the expression of genes involved in cell adhesion, differentiation and cancer testis agents (CTAs) (*Rodríguez-Hernandéz* et al – *2016*).

# 4 Vitamin D

Vitamin D is a fat-soluble vitamin naturally present in some foods and it is synthesised endogenously in the skin cells using the UV sun energy. Activity of vitamin D is done via its active metabolite 1,25-dihydroxy-vitamin D (1,25-$D_3$), also known as calcitriol (*Holick – 2007; Silva* et *Furnaletto - 2018*). Vitamin D plays an important role in bone metabolism by helping $Ca^{2+}$ and phosphate ($PO_4^{3-}$) deposition and homeostasis. In addition, several genes were found to be regulated by calcitriol and vitamin D receptor (VDR). Studies showed that 1,25-$D_3$ is important in autoimmune and cardiac diseases, neurological diseases, diabetes, etc. The main focus of this thesis is to understand the role of CaSR in NB, thus in this chapter it will be discussed how 1,25-$D_3$ affects $Ca^{2+}$ and $PO_4^{3-}$ homeostasis, bone metabolism and cancer development as well as the molecular mechanisms associated with it.

## 4.1 Vitamin D synthesis and mechanisms of action

The main source of vitamin D is exposure to sun-light. Solar ultraviolet B radiation penetrates the skin and transforms the substrate 7-dehydrocholesterol to pre-vitamin $D_3$, which is then converted to vitamin $D_3$ (*Holick – 2007*). Vitamin D obtained from diet or synthesized in skin cells is biologically inert. It becomes an active compound during several steps: First, it is hydroxylated in the liver by mitochondrial and microsomal D-25-hydroxylases (encoded by *CYP27A1*) into 25-hydroxycholecalciferol (25(OH)$D_3$). Then it is 1α-hydroxylated in the kidney by the mitochondrial 1α-hydroxylase into the active 1,25-$D_3$. The rate by which 1,25-$D_3$ is produced is limited by the 24-hydroxylations of 25(OH)$D_3$ and 1,25-$D_3$ by the enzyme 25-hydroxyvitamin D 24-hydroxylase. This chemical reaction leads to the formation of the less active 24,25(OH)$D_3$, that induces the synthesis of 1,25-$D_3$, and calciotric acid, excreted in urine. In addition, $Ca^{2+}$, $PO_4^{3-}$ and 1,25-$D_3$ concentration negative regulate its synthesis (*Deeb* et al – *2007*). PTH also plays a part in the regulation of 1,25-$D_3$ synthesis by inducing the expression of *CYP27B1* (*Brenza* et *DeLuca* – *2000*) (Figure 13).

Genomic actions of 1,25-$D_3$ occurs by its binding to the VDR, a transcription factor and member of the steroid hormone nuclear receptor family. In order of this to happen, the complex 1,25-$D_3$-VDR heterodimerizes with the complex 9-*cis*-retinoic acid X receptor (9-*cis*-RXR) migrating towards the nuclei, where it binds to the

vitamin D response elements (VDREs). VDREs are present in the promoter regions of several genes and are characterized by two hexanucleotide repeats separated by
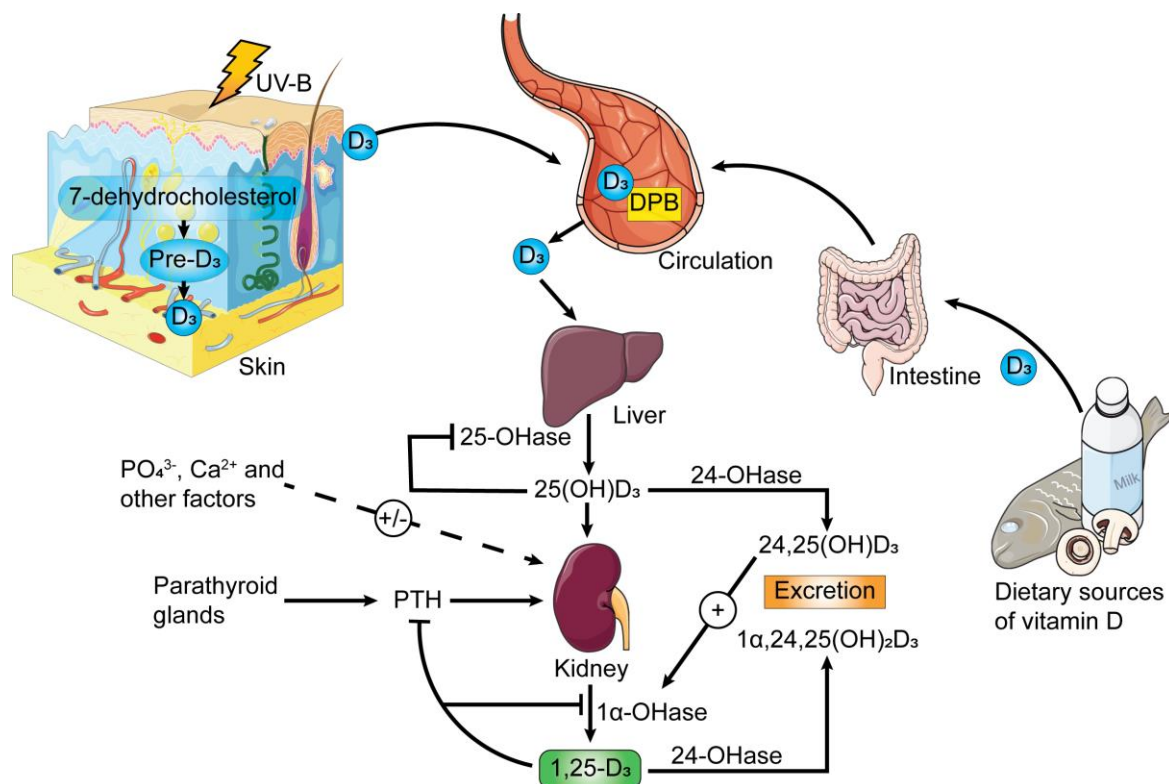


**Figure 13 – Metabolism of 1,25-D₃**. Vitamin $D_3$ can be obtained by diet or by UV-B exposure. This radiation forms pre-vitamin $D_3$ (pre-$D_3$) from the pro-vitamin D (7-dehydrocholesterol). Vitamin $D_3$ ($D_3$) generated from the isomerization of pre-$D_3$ in the skin circulates in the blood stream bound to vitamin D binding protein (DBP). The liver converts vitamin D to 25-hydroxycholecalciferol (25(OH)$D_3$) by the action of 25-hydroxylases (25(OH)ase). Then it is transported to the kidneys where is converted by 25-hydroxyvitamin $D_3$-1α-hydroxylase (1α-OHase) to its active form 1α,25-dihydroxide vitamin $D_3$ (1,25-$D_3$) that exerts several roles in different target cells. PTH, $Ca^{2+}$ and $PO_4^{3-}$ are the main regulators of 1,25-$D_3$ levels working together in various tissues to regulate each other concentrations. 24-hydroxylation of 25(OH)D3 and 1,25-$D_3$ to 24,25(OH)$D_3$ and 1α,24,25(OH)2D3, respectively, by 25-hydroxavitamin D 24-hydroxylase is the rate limiting step of vitamin D metabolism (adapted from *Deeb* et al – *2007*).

three nucleotides (called DR3) (*Carlberg* et al – *1993; Pike* et al – *2012*).

Regulation of gene transcription by the 1,25-D₃-VDR-RXR complex occurs via recruitment of a number of co-factor proteins after complex binding. Recruited co-factors have chromatin modifying abilities that repress or activate gene transcription. Recruitment of steroid receptor coactivators (SRCs), nuclear coactivator-62kDa–Ski-interacting protein (NCoA62–SKIP) and histone acetyltransferases (HATs), CREB binding protein (CBP)–p300 and polybromo- and SWI-2-related gene 1 associated

factor (PBAF–SNF) leads to histone acetylation and transcription activation. Transcription initiation occurs by the binding of vitamin D receptor-interacting protein (DRIP) 205 to activation function 2 (AF2) and the attraction of a mediator complex containing other DRIPs that create a bridge towards the transcription factor 2B (TF2B) and RNA polymerase II (*Haussler* et al – *2013*). Repression of transcription by the 1,25-D$_3$-VDR-RXR happens with its association with VDR-interacting repressor (VDIR) that is bound to E-box-type negative VDREs leading to the dissociation of the HAT co-activator and the recruitment of histone deacetylase (HDAC). This complex set of reactions results in chromatin deacetylation and remodelling and consequent gene repression (*Kim* et al – *2007; Deeb* et al – *2007; Pike* et al – 2012). These processes are tissue specific and gene specific, making
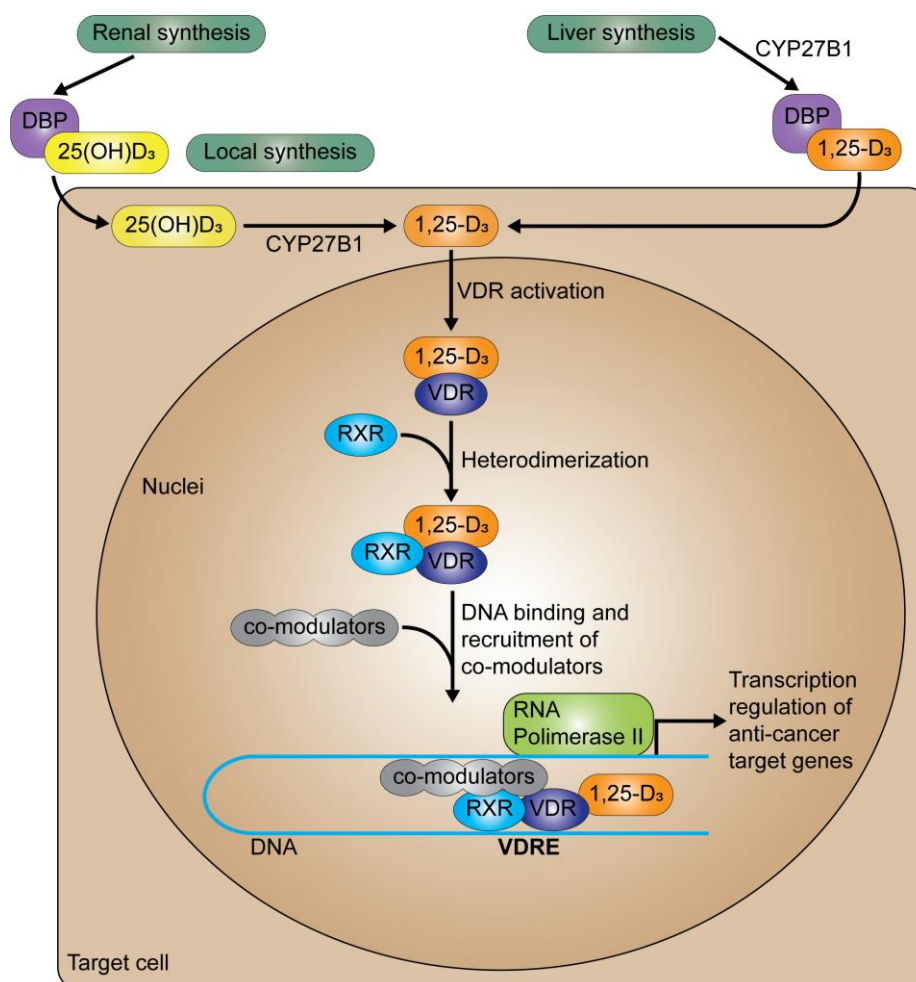


**Figure 14 – Mechanisms of action of 1,25-D$_3$.** After being processed in the cytoplasm, 1,25-D$_3$ exert its action in the nuclei where it binds to its receptor (VDR). Binding to VDREs occurs after heterodimerization with RXR and gene transcription is activated or inhibited when heterodimeric complex binds to co-modulators to coil or uncoil chromatin (*Feldman* et al – *2014*).

the effect that $1,25\text{-}D_3$ highly variable according to cell type (Figure 14).

## 4.2   Role of $1,25\text{-}D_3$ in $Ca^{2+}$ homeostasis

In the previous chapter we described how CaSR is the main player in the maintenance of $Ca^{2+}$ homeostasis. Another key player for this essential physiological process is $1,25\text{-}D_3$. This molecule participates in the regulation of $Ca^{2+}$ and $PO_4^{3-}$ levels by acting on their intestinal absorption, renal excretion, and calcium bone mobilization (*Xue* et *Fleet – 2009*). A reduction of $Ca^{2+}$ in the plasma stimulates PTH secretion and $1,25\text{-}D_3$ synthesis. On the contrary, high $Ca^{2+}$ concentrations activate CaSR, resulting in PTH secretion inhibition and decrease of both $1,25\text{-}D_3$ and $Ca^{2+}$ (*Gil* et al – *2018*). $1,25\text{-}D_3$ has a direct effect in PTH regulation, as it was shown that together with VDR increases the expression of this hormone (*Kim* et al – *2007*). Absorption of nutrients in the intestines occurs via the intestinal epithelium (paracellular) or actively via intestinal cells (transcellular). In the presence of high dietary $Ca^{2+}$, paracellular transport is sufficient which is strictly regulated by $1,25\text{-}D_3$ and occurs to increase $Ca^{2+}$ mobilization (*Xue* et *Fleet – 2009*). This happens in three stages: entrance of $Ca^{2+}$ via $Ca^{2+}$ channels present in the apical side, intracellular transport mediated by calbindin and active $Ca^{2+}$ transport to blood stream in the basolateral membrane (*Wongdee* et *Charoenphandhu – 2015; Gil* et al – *2018*). PTH and $1,25\text{-}D_3$ increase the reabsorption of $Ca^{2+}$ in the renal distal tubes. The transcellular transport of $Ca^{2+}$ has the same steps as in the intestine and it is regulated by $1,25\text{-}D_3$. Reabsorption of $PO_4^{3-}$ in this organ occurs indirectly by increase of FGF-23 and its co-receptor α-klotho (*Perwad* et *Portale – 2011; Glendenning* et al – *2000*).

In the presence of low plasma $Ca^{2+}$ concentration, $1,25\text{-}D_3$ will activate VDR in a PTH-dependent mechanism inducing the formation and differentiation of osteoclasts (*Gil* et al – *2018*). These cells will mobilize $Ca^{2+}$ by activating receptor activator of nuclear factor kappa-κ (RANKL) ligand that is the main player in osteoclast genesis and bone reabsorption. $1,25\text{-}D_3$ increases pyrophosphate and osteopontin leading to the inhibition of bone mineralization (*Nakamishi* et al – *2017*).

## 4.3   Role of Vitamin D in bone formation and mineralization

The organ where vitamin D has a more significant role is in the bone, where both, its receptor and proteins important for the synthesis of $1,25\text{-}D_3$, are expressed

(*van Driel* et *van Leeuwen – 2017*). Mice lacking VDR show growth retardation after weaning and infertility. Additionally, VDR knock-out animals presented low $Ca^{2+}$ and $PO_4^{3-}$ levels and developed alopecia and showed flat the face, a phenotype that is similar to the human disease rickets type II (*Yoshizawa* et al *– 1997*). This is a rare autosomal disease associated with VDR mutations, where organs of the patients are resistant to 1,25-$D_3$ and show bone softening and weakening (rickets) (*Michałus* et *Rusińska – 2018*).

Generation of cell specific knock-out mice allowed for a greater understanding of the specific role of vitamin D in bone. Mice where VDR was absent in osteoblasts, showed an increase in bone mass. This was due to a defect in bone reabsorption and not in bone mineralization (*Nakamishi* et al *– 2017*). Defects in bone mineralization observed in VDR full knock-out were due to the absence of this receptor in intestine, inducing a reduction of $Ca^{2+}$ absorption and increase of bone mineralization. VDR mice supplemented with a high $Ca^{2+}$ diet after weaning showed a less severe phenotype (*Amling* et al *– 1999*).

*In vitro* studies using both single and co-cultures of osteoblast and osteoclasts showed that expression of VDR and 1,25-$D_3$ play different parts in these cells. Hematopoietic cells differentiate into osteoclasts when treated with macrophage colony-stimulating factor (M-CSF) and RANKL (*Nicholson* et al *– 2000*). Osteoclast differentiation from human monocytes was also achieved when these cells were treated with near physiological concentrations of 1,25-$D_3$ (*Zarei* et al *– 2016*). Studies using mouse hematopoietic cells and osteoblast-lineage cells, showed that absence of VDR in osteoblast lineage prevented the differentiation of osteoclast in the presence of 1,25-$D_3$ (*Takeda* et al *– 1999*). It was also shown that in mouse bone marrow macrophages, 1,25-$D_3$ inhibited osteoclast differentiation by inhibiting c-Fos, a transcription factor essential for this process (*Grigoriadis* et al *– 1994; Takasu* et al *– 2006*). Additionally, it was shown that treatment of an osteoblastic human cell line with 1,25-$D_3$ or 24-hydroxylated product increased their differentiation status seen by increase of expression of osteocalcin, alkaline phosphatase and mineralization (*van Driel* et al *– 2006*).

## 4.4 Anti-tumorigenic properties of vitamin D

Vitamin D and VDR play an important role controlling the expression of different genes associated with cellular proliferation and differentiation, suggesting a

key role in cancer prevention. Several studies associated vitamin D deficiency with an increase of cancer risk and evaluating anti-tumorigenic properties of 1,25-D$_3$ or its analogues. Conversely, other studies suggest a link between high vitamin D levels and an increase in cancer risk (*Helzlsouer – 2010*). American Institute of Medicine (IOM) only agrees that adequate amounts of vitamin D are needed to maintain bone mineralization and does not endorse its anti-tumorigenic capacities due to the lack of randomized clinical trials (*Feldman* et al – 2014, *Institute of Medicine Report – 2011*). In spite of this, several studies demonstrated the anti-tumorigenic capacity of 1,25-D$_3$ in adult and childhood tumours.

Using colon cancer cell lines, it was shown that 1,25-D$_3$ has both anti-proliferative and differentiation properties; however, the mechanisms are cell line dependent. In SW480-ADH, 1,25-D$_3$ treatment increased E-cadherin with consecutive increase of β-catenin nuclei translocation and inhibition of Wnt/ β-catenin/TCF-4 signalling inducing cell differentiation (*Pálmer* et al – 2001). Treatment of Caco-2 cells with 1,25-D$_3$ reduced their growth and increased their differentiation status due to an inhibition of the EGF receptor and signalling as well as the transcriptional activation of activator-protein 1 (AP-1) (*Tong* et al – *1999; Chen* et al – *1999*). Apoptosis was increased in HT-29 and SW620 cells due to an increase of the pro-apoptotic protein BAK after 1,25-D$_3$ and its analogue EB1089 (*Diaz* et al – 2000). In addition, *in vivo* preclinical studies using transgenic mouse models, showed that 1,25-D$_3$ treated mice reduced tumour burden and number of polyps. Moreover, this drug also inhibited tumour growth (*Huerta* et al – 2002; *Akhter* et al – *1997*).

Most studies evaluating the effect of vitamin D in breast cancer used the MCF-7 cell line. *In vitro,* it was shown that 1,25-D$_3$ treatment of this cell line reduced proliferation by inhibition of MAPK signalling, BRCA1, survivin and induced apoptosis by increasing BAK nuclei translocation (*Campbell* et al – 2000; *Capiati* et al – 2004; *Narvaez* et al – 2001). Mouse xenograft models were used to evaluate the effect of vitamin D analogues in combination with commonly used chemotherapy agents. It was shown that 22-oxa-calcitriol inhibited breast cancer tumour growth of ER positive and negative xenografts, this effect was exacerbated when combined with tamoxifen (*Abe-Ashimoto* et al – *1993*). Koshizuka and his colleagues tested the effect of three vitamin D compounds alone or in combination with paclitaxel. They

observed that all inhibited tumour growth, and there was an additive effect when they were combined with paclitaxel (*Koshizuka* et al – *1999*).

Several studies demonstrated the anti-proliferative effect of 1,25-D$_3$ in prostate cancer cell lines. This was a result of cell cycle arrest via increase of p27 and decrease of nuclear localization of cyclin dependent kinase 2 (CDK2) (*Yang* et al – *2003*). Additionally, 1,25-D$_3$ induced cell death of prostate cancer cell lines by down-regulation of Bcl-2, Bcl-X and p21 (*Blutt* et al – *2000; Hershberger* et al – *2001*). In a transgenic mouse model of prostate epithelial neoplasia, 1,25-D$_3$ treatment during early stage disease delayed reduced tumour development (*Banach-Petrosky* et al – *2006*). Anti-tumorigenic properties of vitamin D and its analogues were also studied in mouse prostate cancer cell line xenografts, where it was shown that it reduces tumour growth without causing hypercalcemia (*Blutt* et al – *2000, Polek* et al – *2001*).

In pancreatic cancer cells, 1,25-D$_3$ and analogues showed anti-proliferative properties by affecting cyclin expression (such as D1, E and A), cyclin depend kinases (CDKs) and inducing G0/G1 arrest. This hormone also induced p21 and p27, thus increasing of apoptosis (*Kawa* et al – *1996, Zugmaier* et al – *1996*). *In vivo,* the analogue 22-oxa-calcitriol inhibited the growth of a xenograft tumour model more significantly than 1,25-D$_3$ without inducing hypercalcaemia (*Kawa* et al – *1996*).

Response of NB cell lines to 1,25-D$_3$ treatment is cell specific and amplification of *MYCN* influences this response. In addition, NB cell lines show different endogenous levels of VDRs, altering their response to 1,25-D$_3$ and some of its analogues (*Gumireddy* et al – *2003; Lange* et al – *2010*). It was seen that proliferation of the commonly used NB cell line SH-SY5Y was reduced in the presence of 1,25-D$_3$. This resulted from the influence of VDR in the expression and function of ceramide kinases, as well as in the formation of transcription regulator complex VDR/COUP-TFI/HDAC (*Bini* et al – *2012*). It was shown that the analogue 20-*epi*-1α,25-dihydroxidevitamin D$_3$ (20-*epi*-1,25-D$_3$) was more potent than 1,25-D$_3$ in inducing cytotoxicity. This response was cell line dependent, and in those where *MYCN* was amplified treatment with 20-*epi*-1,25-D$_3$ reduced the levels of this oncogene (*Gumireddy* et al – *2003*). *In vivo* studies were conducted using SK-N-SH and SK-N-AS xenografts, and in both models, animals treated orally with 1,25-D$_3$ there was inhibition of tumour volume (*van Ginkel* et al – *2007; Lange* et al – *2010*).

The vitamin D analogue B3CD was more potent *in vivo*, reducing tumour growth and stabilized its volume until the end of the *in vivo* study (*Lange* et al – *2010*).

# II. Hypothesis and Aims

# 5    Hypothesis and Aims

NB is a complex tumour with a wide range of clinical manifestations and biological features. This heterogeneity is reflected in the survival rate of NB patients, ranging from 90% in patients diagnosed with low and intermediate risk disease to less than 50% in patients with high-risk NB (*Whittle* et al – *2017*). Over the years, research efforts have focused on improving event-free and overall survival. In spite of this, patients with relapse and/or refractory tumours still present low survival rates (*Zage* – *2018*). Thus, there is a need to prevent and/or delay these events, increasing patient survival and quality of life (*Matthay* et al – *2016*). Some of the strategies to achieve this rely on the use of differentiating agents (such as 13-*cis*-RA) and/or immunotherapy agents (such as anti-GD2 antibodies and cytokines) (*Whittle* et al – *2017; Matthay* et al – *2016*). CIN, an allosteric activator of CaSR, significantly inhibits high-risk neuroblastoma growth via ER stress-coupled apoptosis and differentiation induction. However, due to the low endogenous expression of CaSR in more aggressive tumours, inhibition of tumour growth is observed only over prolonged exposure to CIN (*Rodríguez-Hernández* et al – *2016*). Additionally, CIN activates CaSR present in the parathyroid glands inhibiting PTH secretion and reducing plasma $Ca^{2+}$ levels, which can be a very debilitating side effect for NB patients (*Nemeth* et al – *1998; Nemeth* et al – *2004*).

In this context, our overall hypothesis is that modulation of expression and activation of CaSR can be a potential therapeutical approach for NB. The present study aims to identify and characterize potential drugs that could improve patient outcome, especially for high-risk NB patients. To fulfil this, the main aims of this work are:

**1.**    Investigate if the anti-tumorigenic effect of CIN can be potentiated or accelerated by its combination with different drugs that increase the CaSR expression levels in high-risk NB:

    **1.1.**    Evaluate the anti-tumorigenic potential of the combined therapy of CIN and vitamin D in its active form 1,25-$D_3$.

    **1.2.**    Evaluate the effect of the combined treatment of CIN and retinoids (ATRA or 13-*cis*-RA).

**2.** Identify and characterize other positive allosteric modulators of CaSR that might exert neuroblastoma-specific or predominant effects without inducing hypocalcaemia in NB patients.

# III. Material and Methods

# 6 Materials and Methods

## 6.1 Cell culture

### 6.1.1 Cell lines

Neuroblastoma cell lines from table 6 were obtained from the repository at the Laboratory of Developmental Tumours of the *Institut de Recerca Pediatrica Sant Joan de Déu* (Barcelona). Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Waltham, NA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA,USA), 2mM L-glutamin, penincilin (100 U/mL) and streptomycin (100 ug/mL) (all from Gibco). Cell cultures were maintained at 37ºC in a humidified atmosphere containing 5%CO2.

| Cell line | Phenotype | *MYCN* status | *TP53* status |
|---|---|---|---|
| **LA-N-1** | N-type | amplified | Mutated |
| **LA1-55n** | N-type | amplified | wild-type |
| **SK-N-SH** | N-type | amplified | wild-type |
| **SH-SY5Y** | N-type | non-amplified | wild-type |
| **IMR32** | N-type | amplified | wild-type |
| **IMR5** | N-type | amplified | wild-type |
| **SK-N-LP** | I-type | amplified | wild-type |
| **SK-N-JD** | I-type | amplified | wild-type |
| **LA1-5S** | S-type | amplified | Mutated |
| **SK-N-AS** | S-type | non-amplified | Mutated |

**Table 6 –** Neuroblastoma cell lines phenotype

### 6.1.2 Reagents

Reagents used are listed in table 7.

| Drug | Company | *In vitro* | | *In vivo* | |
|---|---|---|---|---|---|
| | | Conc. | Vehicle | Conc. | Vehicle |
| **1,25-D$_3$** | Selleck | 100nM | Ethanol | | |
| **CIN** | Selleck | 1µM | DMSO | | |
| **CIN** | Amgen | | | 10mg/kg | 0.5% Tween-20, 0.25% carboxylmethylcellulose |
| **ATRA** | Sigma | 2.5µM | DMSO | 2.5mg/kg | 10%DMSO+90% soybean oil |
| **13-*cis*-RA** | Sigma | 2.5µM | DMSO | 30mg/kg | Soybean oil |
| **AC-265347** | Sigma | 1µM | DMSO | 10mg/kg | 5% DMSO+ 5% ddH$_2$O + 90%PEG400 |

**Table 7 –** Reagents and concentrations used in each assay. Concentration (Conc.)

### 6.2 Induction of CaSR expression

#### 6.2.1 CaSR induction by drugs

LA-N-1 or IMR5 cells were seeded in 10-cm petri-dishes ($1 \times 10^6$) and 24-hours later drugs were added. Cells were in contact with 1,25-$D_3$, ATRA or 13-*cis*-RA during 8-, 24-, 48- or 72-hours, after this time cells were harvested by scraping and collected in a 15mL tube. After washing with 1mL of PBS, pellet was resuspended in 1mL of TRizol® (Thermofisher, Waltham, MA, USA) to extract RNA, or in RIPA buffer (see section 3.11.3) complemented with protease inhibitor cocktail (Roche, Basel, Switzerland), for protein extraction.

#### 6.2.2 Stable transfection of CaSR

IMR5($0.75 \times 10^6$) and LA-N-1($0.5 \times 10^6$) cells were seeded in 6-well plates and 24-hours later, cells were transfected with 2.5µg of pCMV-GFP or pCMV-CaSR-GFP (Origene, Rockville, MD, USA) using Lipofectamine (Invitrogen) according to manufacturer's recommendations. 48-hours post-transfection, media was removed, and selection antibiotic was added, 250µg/mL for LA-N-1 and 50µg/mL for IMR5 of G418 (Sigma). After 6 weeks selection *CaSR* mRNA levels were monitored by real-time quantitative PCR (RT-PCR) and protein levels were quantified by immunoblot.

### 6.3 Cell viability assay

Assays were conducted by seeding 1000 (LA-N-1) or 2500 (IMR5) cells in each well of a 96-well plate. To evaluate the effect of combination of different drugs with CIN, cells were first treated with 100nM of 1,25-$D_3$, 2.5µM of ATRA or 13-*cis*-RA for 24-hours. After this, 1µM of CIN was added and cell viability was evaluated at day 2, 3, 4 and 5 after adding CIN. For assays evaluating effect of calcimimetics in cells overexpressing CaSR, 1µM of AC-265347 or CIN was added to cells 24-hours after seeding and cell viability was quantified after 3, 4, 5 and 6 days of treatment. Quantification of cell viability was done by adding 10µl of CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS, Promega) to each well, and incubated for 3-hours in a humidified incubator at 37ºC, 5% $CO_2$. Absorbance measured at 490nm using a TECAN INFINITE FNano⁺ (Männedorf, Switzerland) was directly proportional to the number of living cells in each well.

### 6.4 Differentiation assay

#### 6.4.1 Combination of vitamin D or retinoids with CIN

LA-N-1 (0.5x106) or IMR5 (0.5x106) were seeded in a 10-cm dish and the following day cells were treated with 100nM of 1,25-D3, 2.5µM of ATRA or 2.5µM 13-cis-RA. After 24-hours of treatment media was removed and cells received fresh media containing either of these drugs alone or in combination with 1 µM of CIN. Cells received fresh nutrients and drug every three days and were split using trypsin, when confluence was higher than 80%. Cells were collected by scraping, to evaluate mRNA or protein expression after 12 days of treatment.

#### 6.4.2 Chronic exposure to calcimimetics

LA-N-1 ($0.5x10^6$) or IMR5 ($0.5x10^6$) were seeded in a 10-cm dish and the following day cells were treated with 1µM of CIN, 1µM AC-265347 or their vehicle (0.00002% DMSO). Cells received fresh nutrients drug every three days and were split using trypsin, when confluence was higher than 80%. Cells were collected to evaluate mRNA or protein expression after 14 days of treatment.

### 6.5 Colony formation assay

IMR5 (2500) and LA-N-1 (1000) cells were seeded in 6 well plates previously coated with 1mg/mL of fibronectin (Sigma). The following day, cells were treated with ATRA, 13-*cis*-RA (both 2.5µM) or 1,25-D3 (100nM) alone. Then, CIN was added and cells were maintained in this media until colonies were visible to the naked eye (10-12 days, depending on the cell line). At this point, media was removed, and cells were washed with PBS and then fixed with 4% paraformaldehyde (PFA) at room temperature (RT) for 15 min. After fixation, cells were washed with PBS and stained with crystal violet solution (2% w/v, 20% methanol in PBS) at RT, for 2 min. Then, plates were rinsed with tap water to eliminate crystal violet excess. Images were captured in the Audiovisual Unit of Hospital Sant Joan de Dèu (HSJD) and quantification of number of colonies was done using Strata Quest (version 6) (TissueGnostics GmbH, Vienna, Austria).

### 6.6 Half inhibitory concentration (IC$_{50}$) assay

To determine the concentration of drug required to induce a reduction of 50% of cell viability (IC$_{50}$), assays were conducted by seeding 5000 cells in each well of a 96-well plate. The following day, six technical replicates were treated for each of

the following concentrations of AC265347: 100µM, 30 µM, 10 µM, 3 µM, 1µM, 300nM, 100nM, 30nM, 10nM and 0nM. After 72-hours, cell viability was quantified by adding 10µl of CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay to each well. Cells were incubated for 3-hours in a humidified incubator (37°C, 5% $CO_2$) and then, absorbance was measured at 490nm using a TECAN INFINITE FNano$^+$. $IC_{50}$ was calculated using GraphPad Prism 7 software (GraphPad).

## 6.7 Acute exposure to calcimimetics

LA-N-1 and IMR5 cells were seeded in 10cm petri-dishes (for immunoblot, $1.5 \times 10^6$ cells) or 6-well plate (for annexin V staining, $0.5 \times 10^6$). The following day media cells were synchronized by replacing RPMI with calcium free Dulbecco's modified Eagle media (DMEM) (Gibco) supplemented with bovine serum albumin (BSA) (0.2% w/v), 4mM L-glutamin, 2mM penincilin-streptomycin and 0.5mM $CaCl_2$. After an overnight starvation, cells were treated with DMSO (0.001%), AC-265347 (1µM) or CIN (1µM) in the presence of 0.5mM $CaCl_2$ or 3mM of $CaCl_2$. For evaluation of cell death using immunoblot, cells were collected by scraping after 24-hours of stimuli. Quantification of cells death using annexin V staining was done by collecting cells after 16-hours of treatment.

## 6.8 Flow cytometry analysis

### 6.8.1 Cell cycle analysis

LA-N-1 and IMR5 ($0.5 \times 10^6$) cells were seeded in 10cm petri-dishes and the following day 1µM of AC-265347 or CIN was added. Every 3 days fresh medium containing new drug was added. After 14 days of treatment, cells were harvested using trypsin and pellet was resuspended in 0.5mL of PBS and 4.5mL of ice-cold ethanol. Then, cells were put on ice and washed twice with ice cold PBS. Nuclei staining was done with a solution of 1mg/mL of propidium iodide (Sigma) at RT, for 30-min. Acquisition of cells was done immediately after using a Novocyte Flow cytometer equipped with NovoExpress software (ACEA Biosciences, CA, USA). Analysis of flow cytometry data was performed in FlowJo (Becton, Dickinson & Company, Franklin Lakes, NJ, USA).

### 6.8.2 Annexin V apoptosis assay

Cells were collected from 6-well plate using trypsin and washed with PBS. After centrifugation at 300g, pellets were resuspended in 50µL of annexin $Ca^{2+}$ buffer

containing 5% Pacific Blue Annexin V and 1% SYTOX® Green (Invitrogen) and incubated for 20-min a room temperature (RT), according to manufacturers specifications. After incubation, 200μL of Annexin V buffer (supplied by the manufacturer) were added to each sample and acquisition was done in Novocyte Flow cytometer and analysed with the NovoExpress (ACEA Biosciences). Analysis of flow cytometry data was performed using FlowJo (Becton, Dickinson & Company, Franklin Lakes, NJ, USA).

## 6.9   Molecular Biology techniques

### 6.9.1   RNA extraction

RNA isolation of xenografts, a piece of frozen tumour (approximately $1mm^3$) was cut and put in a safe-lock microtube (Eppendorf, Hamburg, Germany) containing stainless steel beads 0.9-2mm (Next advance, Troy, NY, USA) and 300μL of TRIzol™ (Thermofisher). Tissue was homogenised using a Bullet Blender storm 24 (Next advance) at maximum speed for 2-5min until a homogenous mixture is obtained. Homogenous tissue mixture is transferred to a screw-cap tube and 700μL of TRIzol™ and RNA isolation was done according to manufacturer's specifications. Alternatively, RNA extraction from cell pellet was performed resuspending cells in in 1mL of TRIzol™ and RNA was isolated according to manufacturer's recommendations. Briefly, 200μL of chloroform were added to 1mL of TRIzol, after 8-min incubation, tubes were centrifuged at 12000rpm for 10 min. Top-phase was collected and put in 500μL isopropanol, this was then incubated and centrifuged at 12000rpm for 8 min. Pellet was resuspended in 75% ethanol and centrifuged for 5 minutes at 12000rpm, after thorough removal of the ethanol, pellet was dissolved in water. RNA was measured in Spectophotometer NanoDrop™ 2000 (Thermo Scientific, Waltham, MA, USA).

### 6.9.2   Quantitative reverse transcription PCR (RT-qPCR)

Purified RNA (1μg) was reverse-transcribed into first strand complementary DNA (cDNA) using random primers and M-MLV (Promega). RT-qPCR was done using gene specific assay on demand and TaqMan® universal PCR master mix or specific primers and SYBR Green (table 3) (Applied Biosystems, Forster City, CA,USA) in a QuantStudio 6 Flex (Applied Biosystems). Calculation of gene relative expression was done using the $2^{-\Delta\Delta Ct}$ method and the *TATA-box binding protein*

(TBP) as a house keeping gene. Quality of sample and cDNA was assured when Ct of TBP was lower than 30.

### 6.9.3 Protein extraction and Immunoblot

A piece of frozen tumour (approximately $1mm^3$) was cut and put in a safe-lock microtube (Eppendorf) containing stainless steel beads 0.9-2mm (Next advance) and 100-150µL of RIPA buffer (10mM Tris-HCl pH6.8, 1mM EDTA, 150mM NaCl, 1%SDS) in the presence of a proteinase inhibitor cocktail (Roche). Tissue was homogenised using a Bullet Blender storm 24 (Next advance) at maximum speed for 2-5min until a homogenous mixture is obtained. Homogenous cell solution was transferred to a microtube and incubated for 15 min at 4°C, after incubation tubes were centrifuged for 15min at 12000rpm and supernatant was collected. Alternatively, cell pellet was thoroughly resuspended in an equal volume of RIPA buffer and in incubated for 15min at 4°C, protein was obtained after centrifugation for 15min at 12000rpm.

Protein for each sample was quantified using Bradford assay (BioRad, Hercules, CA) and forty to seventy-five micrograms of protein were mixed 1:5 with Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromphenol blue, 0.125M TrisCL ph6.8, 10% DTT for reducing conditions) and electrophoresed in 8-12% SDS-PAGE gels and transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). After blocking with 3% bovine serum albumin (BSA, Sigma) or skim fat milk (Sigma), membranes were incubated overnight with the antibodies in Table 8 at the indicated concentrations.

| Target | host | Clone | Company | dilution |
|--------|------|-------|---------|----------|
| **CaSR** | mouse | ADD | Abcam | 1:500 |
| **VDR** | rabbit | D2K6W | Cell Signalling | 1:1000 |
| **NF68** | rabbit | T.400.5 | Thermofisher | 1:1000 |
| **TrkA** | rabbit | Y32Ex | Santa Cruz | 1:500 |
| **Tubulin** | mouse | B-5-1-2 | Sigma | 1:30000 |
| **RARα** | rabbit | E6Z6K | Cell Signalling | 1:1000 |
| **RARγ** | rabbit | D3A4 | Cell Signalling | 1:1000 |
| **RXRα** | rabbit | D6H10 | Cell Signalling | 1:1000 |
| **c-Parp** | rabbit | D64E10 | Cell Signalling | 1:1000 |

**Table 8 –** Antibodies used in immunoblots.

### 6.10 *In vivo* studies

All *in vivo* experimental procedures were performed according to the European guidelines (EU directive 2010/63/EU) and were approved by the Institutional Animal Research Ethics Committee.

### 6.10.1 Drug preparation

Cinacalcet used for *in vivo* studies was prepared from tablets of Mimpara® (Amgem, Thousand Oaks, CA). After removing the protective layer, the tablet was homogenized using a mortar and pestle. Powder was weighted and aliquoted in tubes and kept at 4°C until the day of administration. CIN was reconstituted with 0.5% Tween-20 (20%v/v) and carboxylmethylcellulose (0.25%w/v) (both from Sigma) for a final concentration of 20mg/mL. AC-265347 was dissolved in 5% DMSO, 5% water and 90% PEG400 (all from Sigma) for a final concentration of 20mg/mL. Both drugs were administered by oral gavage. ATRA was dissolved in soybean oil (Thermofisher) to have a final concentration of 2.5mg/mL and injected intraperitoneally. 13-*cis*-RA was dissolved in soybean oil to have a final concentration of 7.5mg/mL and administered orally.

### 6.10.2 Establishing in LA-N-1 xenografts

LA-N-1 cells were thawed and expanded 10 days before inoculation day (cells had no more than 3 passages). On inoculation day, cells were collected using trypsin and live cells were counted using a Neubauer chamber and trypan blue (Sigma). Cells suspension was resuspended in PBS to a concentration of $7.5\text{-}10\times10^6$ cells per 100μL and mixed with the same volume of Matrigel (Beckton Dickinson). Four to six-week female athymic nude-Fox1 *nu/nu* mice were subcutaneously injected with 200μL of this solution, which contained $7.5\text{-}10\times10^6$ cells. Palpable tumours appeared approximately 7-10 days after inoculation and started to be measured with a digital calliper (Thermofisher) three times per week. When one or both tumours reached 150-200mm3 mice were randomly assigned to each treatment group. Dimensions of tumours were calculated with the formula L x $W^2$, where "L" is refers as length in mm, and "W" as width in mm.

### 6.10.3 Establishing patient derived xenograft models (PDX)

Patient derived models HSJD-NB001, HSJD-NB004 and HSJD-NB012 were generated by the group of Dr. Angel M. Carcaboso from patient biopsies obtained at

diagnosis or during different treatment stages at the *Hospital Sant Joan de Déu* (HSJD) in Barcelona (Monterrubio et *al*-2015). Following an IRB-approved protocol, fresh (HSJD-NB012) or frozen (HSJD-NB001, HSJD-NB004) tumour fragments were transplanted into the flank of two four to six-week female athymic nude-Fox1 *nu/nu* mice to expand viable tissue. Tumours were measured three times per week with a calliper, and when they reached a volume of 1500mm$^3$, mice were sacrificed by cervical dislocation, and tumours were excised. Visible necrotic areas were removed, and viable tissue was cut into 4-5mm$^3$ pieces and implanted subcutaneously into the flank of four-six-week-old athymic nude mice (15-20 animals per tumour model). When tumours were palpable, they were measured three times per week, and mice were randomly assigned to a treatment group when tumour's volume was between 150-200mm$^3$. Dimensions of tumour were calculated with the formula L x W$^2$, where "L" is refers as length in mm, and "W" as width in mm. Experiments were conducted with fragments at generation 2 (HSJD-NB001), generation 9 (HSJD-NB004) and generation 10-11 (HSJD-0012).

### 6.10.4 Induction of CaSR in LA-N-1 xenografts

LA-N-1 xenografts were generated as explained in section 3.12.2. Mice were treated with 2.5mg/kg of ATRA or their vehicle (VEH) intraperitoneally daily (five times per week). Tumour volume was measured three times per week and mice were sacrificed after one, two or three weeks of treatment. Tumours were excised and a representative part of the tumour was cut for histological analysis (explained in section 3.12.8), the remaining tissue was frozen for posterior mRNA analysis as explained in section 6.9.1 and 6.9.2.

### 6.10.5 Preclinical efficacy of combination of ATRA with CIN

Generation of LA-N-1 xenografts was done as explained in section 6.10.2. Two individual experiments were conducted to evaluate the efficacy of combination of ATRA with CIN. In the first experiment animals were treated daily with 2.5mg/kg of ATRA (given intraperitoneally) alone or in combination with 10mg/kg of CIN (given orally) five times per week. In the second experiment animals received ATRA once or three times per week, alone or in combination with CIN; name of treatment groups and posology is summarized in table 9. Control animals received ATRA's vehicle intraperitoneally and CIN's vehicle orally. Tumours were measured three times per week and mice were sacrificed when tumour reached 2000mm$^3$ or animals were in

treatment for 10 weeks. Animals that lost more than 20% of their initial body weight, or presented a dilated abdomen were sacrificed for ethical reasons before end volume was reached. For EFS curves, event was defined as tumour reaching $1000mm^3$, for first experiment and as tumour reaching $1500mm^3$ for second experiment.

| Treatment group | ATRA posology (intraperitoneal) | CIN posology (oral administration) |
|---|---|---|
| ATRA | 2.5mg/kg five times per week | |
| ATRA+CIN | 2.5mg/kg five times per week | 20mg/kg five times per week |
| ATRA 1d | 2.5mg/kg once a week | |
| ATRA 1d+CIN | 2.5mg/kg once a week | 20mg/kg five times per week |
| ATRA3d | 2.5mg/kg three times per week | |
| ATRA3d+CIN | 2.5mg/kg three times per week | 20mg/kg five times per week |
| CIN | | 20mg/kg five times per week |
| VEH | Soybean oil three times per week | CIN vehicle five times per week |

**Table 9 –** Treatment groups of preclinical efficiency of combination of ATRA and CIN.

Tumours were measured three times per week and mice were sacrificed when tumour reached $2000mm^3$ or animals were in treatment for 10 weeks. Tumours that had palpable size were collected for histological analysis.

### 6.10.6 Preclinical efficacy of combination or 13-*cis*-RA with CIN

LA-N-1 xenografts were generated as explained in section 6.10.4. Mice were treated five times per week with 30mg/kg of 13-*cis*-RA alone or in combination with 10mg/kg of CIN, both given orally. Control animals received 13-*cis*-RA vehicle and CIN vehicle orally. Tumours were measured three times per week and mice were sacrificed when tumour reached $2000mm^3$ or animals were in treatment for 10 weeks. Mice that lost more than 20% of their initial body weight or total tumour burden was higher than $3000mm^3$ (in the case that tumour from one flank grows faster than the other) were sacrificed for ethical reasons. Tumours were collected for histological analysis, for EFS curves event was defined as "tumour reaching $1500mm^3$".

### 6.10.7 Calcemia study

Four to six-week old healthy female athymic nude-Fox1 *nu/nu* mice were treated with 10mg/kg/day of AC-262347 or CIN by oral gavage five times per week. Blood samples were collected from tail-vein 4-hours after treatment. Total $Ca^{2+}$ levels were measured once per week using an EPOC reader (Alere Healthcare, Waltham, MA).

### 6.10.8 Preclinical efficacy of calcimimetics in NB xenograft

Generation of LA-N-1 xenografts was performed as explained in section 6.10.4, and PDX models were created as explained in 6.10.5. Mice received 10mg/kg of AC-265347 or CIN or the vehicle of AC-265347 (Table 2). Measuring of tumours was done three times per week and mice were sacrificed when tumours reached 2000mm$^3$ or animals were in treatment for 10 weeks. Mice that lost more than 20% of their initial body weight or total tumour burden was higher than 3000mm$^3$ (in the case that tumour from one flank grows faster than the other) were sacrificed for ethical reasons. Tumours were collected for histological analysis and mRNA expression analysis. For EFS curves, event was defined as tumour reaching 1500mm$^3$.

### 6.10.9 Tumour histology

Tumours were collected when mice were sacrificed, and a transversal section was included in a histology cassette and fixed in 4% PFA for at least 3-hours. Dehydration was done in ascending concentration of ethanol: 2 min at 70%, 3 min at 96% and 1-hour at 100%. Cassettes with tumour section were then incubated in isopropanol for approximately 5-hours and after drying, they were included in paraffin during 4-hours and 20 min. Paraffin embedded samples of 4-6mm thick were sectioned using a microtome (*de Torres* et al – *2009*) and include in histological slides (Leica, Wetzlar, Germany). Before each staining, sections were deparaffinized by incubating slides in a hot chamber for at least 30min, and then in a Bond dewax solution (Leica) at 72°C for 30 min. After this step, slides were incubated in descending ethanol solutions: 3min at 100%, 3min at 96%, 3min at 70% and 3 min at 50%. Slides were dried before each staining or immunohistochemistry (IHC) for the different protein markers. Heamatoxilin & Eosin (H&E) staining was done in Tissue-

Tek Prima® automated slide stainer using an H&E stain kit (both Sakura Finetek, CA, USA).

### 6.10.9.1 Masson's thrichrome

Slides were incubated in a hematoxylin buffer for 10 min and then washed in running water for a few seconds. Next, slides were stained with Mallory red (Sigma) for 30 min, washed and then incubated in phosphotungstic acid (Sigma) for 3 minutes. Immediately, slides were stained with a solution of light green (Sigma) for 30 min and washed with running water. After dehydration with ascending concentration of ethanol (70%, 96% and 100%) and xylene, for 3 min, tissue slides were mounted with a coverslip using the Tissue-Tek coverslipper (Sakura Finetek). In this staining keratin fibres and erythrocytes were stained in red, collagen fibres were displayed in green and cell nuclei in blue.

### 6.10.9.2 Immunohistochemistry (IHC)

After dehydration slides were pre-treated with a solution of pH=9 (for anti-Ki67) or pH=6 (anti-CaSR, anti-TrkA, anti-CD44, anti-NF68) for 20 min. After this, slides were blocked for 5-min with Peroxidase and for antibodies with mouse host (CaSR and CD44) there was an extra blocking step of 30-min with Goat F(ab) anti-mouse IgG H&L (Abcam). After washes with bond wash solution (Leica) slides were incubated in a wet chamber with the primary antibodies from table 10 at the indicated dilution and for the indicated times. After the incubation with the primary antibody, slides were washed with bond wash solution and then incubated for 8-min with the secondary antibody (anti-mouse or anti-rabbit pre diluted, Novusbio, Littleton, CO, USA). After the incubation, slides were washed and IHC was developed with 3,3'-diaminobenzidine (DAB) (Sigma) until signal was visible and a counter staining with hematoxylin was done. Coverslip mounting was done using Tissue-Tek coverslipper (Sakura Finetek).

| Target | host | Clone | Company | dilution | incubation |
|--------|------|-------|---------|----------|------------|
| **Ki67** | rabbit | 170SS | Novusbio | pre-diluted | 30min, 25°C |
| **CaSR** | mouse | ADD | Abcam | 1/100 | Overnight, 4°C |
| **CD44** | mouse | 156-3C11 | Thermofisher | 1/350 | 30min, 97°C |
| **NF68** | rabbit | T.400.5 | Thermofisher | 1/200 | 30min, 97°C |
| **TrkA** | rabbit | Y32Ex | Santa Cruz | 1/20 | 30min, 97°C |

**Table 10 –** Antibodies used for IHC of mouse xenograft tumours

### 6.10.9.3        Imaging and Quantification

Bright field images of the IHC of the last 6 mice treated with CIN, AC-265347 or their vehicle were acquired in an Axio Imager Z2 attached to a TissueFAXS Slide Loader tissue cytometer and equipped with Tissue FAXS software (version 5.3) (TissueGnostics GmbH) using an EC-Plan Neofluar 20×/0.5 objective. Acquisition of whole tumour area was done automatically with acquisition of 5 x 5mm fields of view (FOVs) with automatic focus. Stitching between each FOV was done automatic, to generate an image of the whole tumour tissue. Quantification of IHC was done using STRATA Quest (TissueGnostics) by detecting positive DAG signal. Analysis algorithm was as created together with Robert Nica, the app developer from TissueGnostics. Quantification of positive cells was challenging due to the compact structure of neuroblastoma tumours. The percentage of positively stained areas was recorded, as an alternative to individual cells.

After colour segmentation (separation of blue and brown signals for IHC and red, blue and green signal for Masson's Trichrome), image was converted to a black and white image to improve differentiation of colour intensities. To avoid recording unspecific binding of antibodies to necrotic areas, the algorithm differentiates between high, medium and low intensity signal (intensity criteria was defined by user for each IHC antibody). Medium and low intensity areas were used to quantify the positive signal for each tumour. Percentage of positive signal was calculated as follows: ("Medium intensity area" + "Low intensity area") / "Total tissue area".

### 6.11  Statistical analysis

All statistical analysis was done using GraphPad Prism, version 7.01 for Windows (GraphPad Software, San Diego, CA, USA).  Statistical analysis between two groups was calculated using a Mann-Whitney test, a one-way ANOVA with a Dunn's correction test was used when three or more groups were compared. EFS survival curves were compared using Log-rank statistics with a Bonferroni correction test for multiple comparisons, significance was considered when $p<0.05$. Gene expression analysis and IHC quantifications from mouse xenograft models were compared using one-way ANOVA with a Dunn's post correction test for multiple comparisons.

# IV. Results

# 7    Combination of 1,25-D₃ and CIN in the treatment of neuroblastoma

We first described that low expression of CaSR in high-risk tumours reduces efficacy of CIN in the treatment for NB (*de Torres* et al – *2009, Rodríguez-Hernández* et al – *2016*). Thus, we hypothesised that increasing CaSR expression in NB would increase CIN efficacy. Calcitriol (1,25-D₃) is the active form of vitamin D and it was described to induce an increase of CaSR expression in colon cancer cell lines by binding and activating VDR, which in turn binds to VDREs in the CaSR gene promoter (*Fetahu* et al – *2014*). Therefore, we hypothesised that a combined treatment with 1,25-D₃ might increase the anti-tumorigenic capacities of CIN in NB by increasing CaSR expression.

## 7.1   Treatment of two NB cell lines with 1,25D₃ increases CaSR expression

In order to explore whether 1,25-D₃ increases the expression levels of CaSR, we first treated two NB cell lines with a low endogenous expression of this GPCR, LA-N-1 and IMR5, with 100nM of 1,25-D₃ for the indicated times and quantified *CaSR* mRNA and protein expression levels.

Treatment of LA-N-1 with 1,25-D₃ for 24-hours increased *CaSR* mRNA expression in two out of three assays (2- and 4- fold increase), although high variability between assays was observed (Figure 15A). Additionally, we evaluated CaSR protein levels by immunoblot in non-reducing conditions, which allowed to detect the mature glycosilated monomere form of CaSR (at approximately 130 kDa) and the immature non-glycosilated CaSR (at approximately 100 kDa). In accordance with mRNA expression levels, quantification of the immunoblot revealed an increase in both immature and mature forms (10- and 5-fold increase, respectively) (Figure 15B).

Treatment of IMR5 cells for 8-hours with 1,25-D₃ resulted in a 5-fold *CaSR* mRNA increase relative to DMSO treated cells. No changes were observed after 24- or 48- hours of treatment (Figure 15C). However, experiments using IMR5 cells also presented high variability. Quantification of CaSR immunoblot of IMR5 treated with 1,25-D₃, showed a 2-fold increase of both the immature and mature CaSR when cells were treated for 48 hours (Figure 15D).
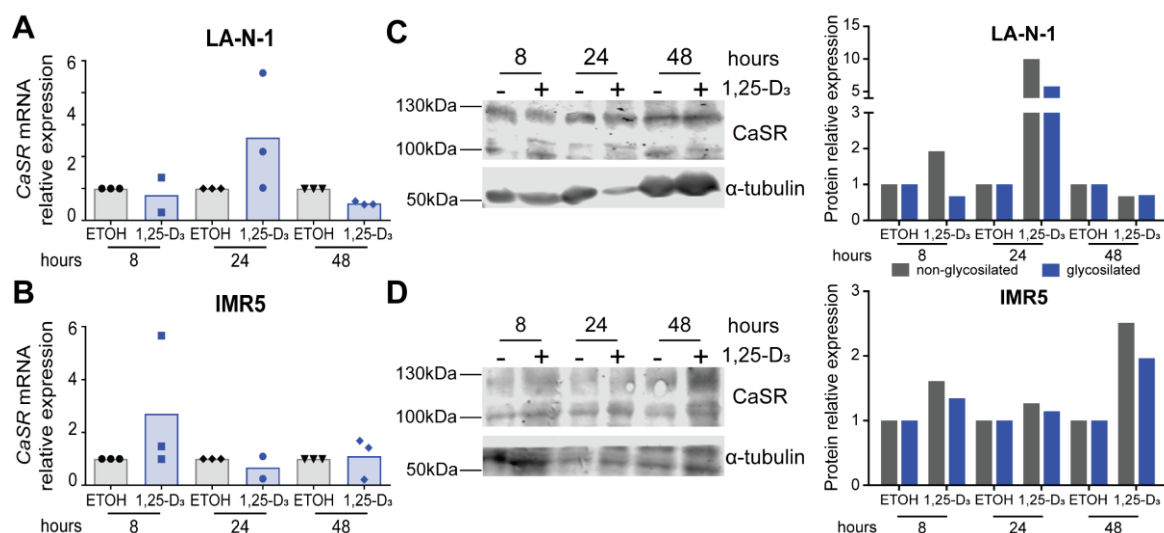
**Figure 15 – Expression of CaSR in LA-N-1 and IMR5 treated with 1,25-D₃. (A-B)** LA-N-1 and IMR5 cells were treated with 100nM of 1,25-D₃ or the vehicle (ETOH) for the indicated times and *CaSR* mRNA expression was quantified by RT-qPCR. Each point represents an individual biological replicate. **(C-D)** Immunoblot showing CaSR expression in LA-N-1 and IMR5 cells treated for the indicated timepoints with 100nM 1,25-D₃. Band intensities were normalized relative to α-tubulin, and the ratio between each CaSR/β-tubulin signal is indicated under the immunoblot. Blots shown are representative of three independent experiments.

## 7.2   Combination treatment of 1,25-D₃ and CIN has a mild effect in LA-N-1 and does not affect IMR5 cell viability and colony formation capacity

To evaluate if 1,25-D₃ would be an interesting combination with CIN, we studied the effect on cell viability and colony formation capacity of NB cell lines. During the first days of treatment, viability of LA-N-1 cells was similar between treatment groups. However, combination of 1,25-D₃ and CIN significantly decreased LA-N-1 cell viability after 5 days of treatment (p=0.0007) (Figure 16A, left panel). On the contrary, viability of IMR5 was unaffected by treatment with 1,25-D₃ and CIN alone or in combination (Figure 16A, right panel).
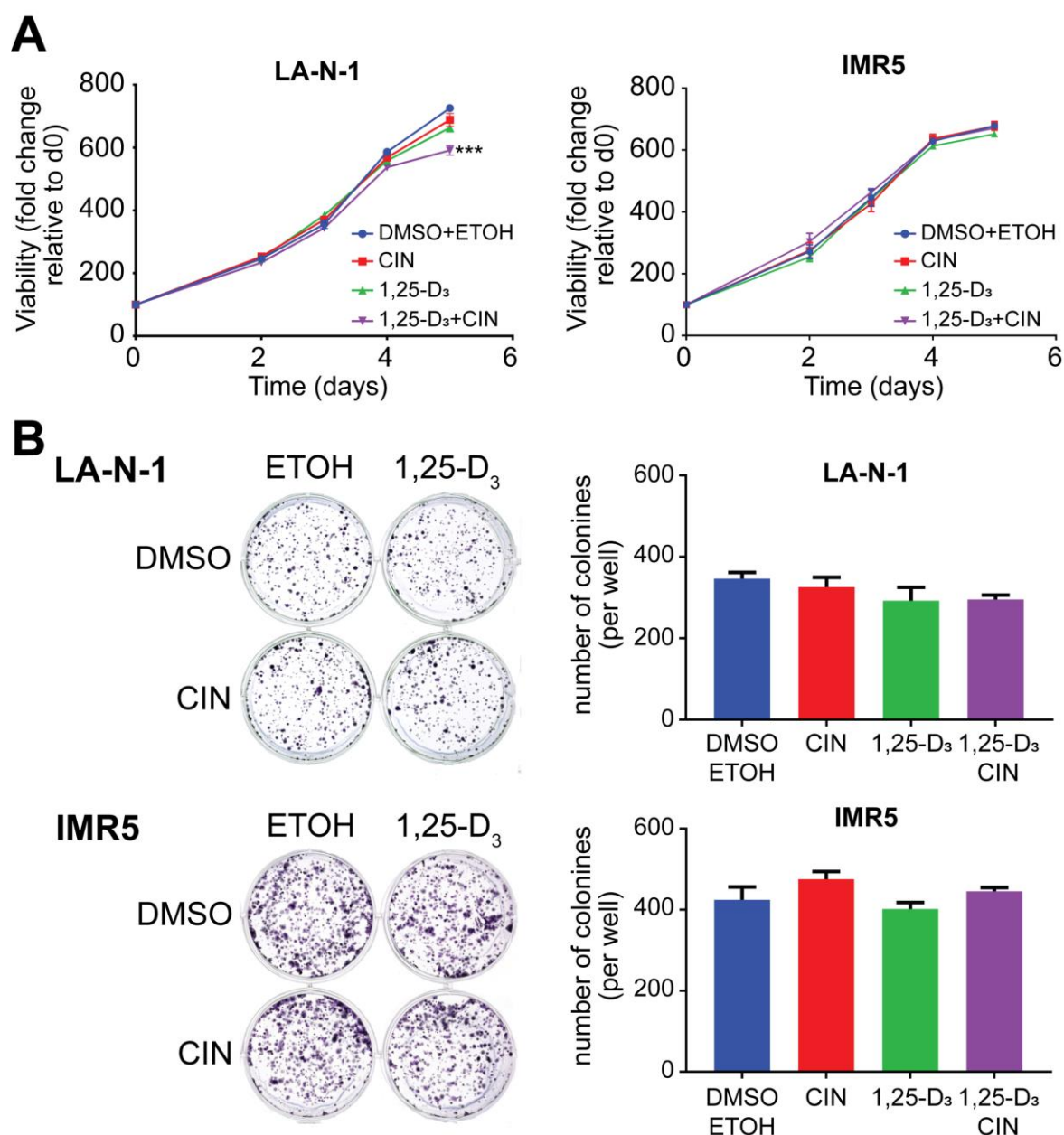
**Figure 16 – Combination of CIN and 1,25-D$_3$ decrease cell viability of LA-N-1. (A)** LA-N-1 and IMR5 cells were treated with 100nM of 1,25-D$_3$ for 24-hours before adding 1μM of CIN. Cell viability was measured using MTS assays after 2, 3, 4 and 5 days of treatment. Each point shows the mean ± SEM of six technical replicates and represents three independent experiments. Statistical significance between treatment groups at each time-point was calculated using a One-way ANOVA with a Dunn's multiple comparison test, ***p<0.001 relative to DMSO. **(B)** LA-N-1 and IMR5 were treated for 24-hours with 100nM of 1,25-D$_3$ before 1μM of CIN was added. After 12 days of treatment colonies were fixed and stained with crystal violet. Bars represent number of colonies that were counted using STRATA Quest in three technical replicates, error bars represent SEM.

Next, we evaluated the effect that the combination of CIN and 1,25-D$_3$ had in the ability to form colonies. This cologenic assay is an *in vitro* cell survival assay

based on the ability of a single cell to grow into a colony, allowing a prolonged drug exposure time. After 12 days of treatment with CIN and 1,25-D$_3$ alone or in combination, LA-N-1 cells maintained their capacity of forming colonies independent of treatment. Consistent with the decrease in cell viability, quantification of the number of colonies showed a reduction of 15% when treated with the combination (Figure 16B). However, in IMR5 cell line, CIN increased by 12% the ability to form colonies, although this was not statistically significant (p=0.7542, DMSO vs CIN), while treatment with 1,25-D$_3$ alone reduced this by 12% (p=1, DMSO vs 1,25-D$_3$). Considering these results, it is no surprising that cells treated with the combination presented higher number of colonies than the control DMSO treated cells (Figure 16B, lower panel).

### 7.3 CIN induces the expression of NB differentiation genes in LA-N-1, and this is abrogated when combined with 1,25-D$_3$

Another strategy to improve prognosis of NB patients is the induction of differentiation of tumour cells (*Whittle* et al – *2017*). We first published that prolonged *in vitro* exposure to CIN induces cytodifferentiation in NB cells (*Rodríguez-Hernández* et al – *2016*). Thus, we studied the effect of CIN and 1,25-D$_3$ had in LA-N-1 cell differentiation by treating them for 12 days with these drugs individually or in combination. Cells treated did not manifest morphological changes, such as neurite outgrowth, that would indicate a higher differentiation status (Figure 17A). First, we evaluated the effect of the treatments in *CaSR* mRNA expression. Although treatment with either 1,25-D$_3$ or CIN alone increased the expression of *CaSR* (1.9- and 3.7-fold, respectively), their combination lead to 8-fold reduction. Additionally, quantification of mRNA expression of genes associated with NB differentiation and aggressiveness, showed that treatment of LA-N-1 with 1,25-D$_3$ increased more than 2-fold the mRNA expression levels of *S100β*, a marker for glial and Schwannian cells of the nervous system (*Sugimoto* et al – *1988*). As we previously described, the treatment of the cells with CIN alone increased the mRNA expression of three NB differentiation markers *TUBB3* (*Guo* et al – *2010*), *TrkA* (*Eggert* et al – *2000*) and *CD44* (*Shtivelman* et *Bishop* – *1991*). Concomitantly there was an 80% reduction mRNA expression levels of *Snail2*, gene encoding for the transcription factor SNAI2 involved in the migration of neural crest cells and has been described to promote NB invasion and metastasis (*Taneyhill* et al -*2007, Vitali* et al - *2008*). Treatment of LA-

N-1 cells with both 1,25-D$_3$ and CIN lead to an increase of *CD44* and *TUBB3* mRNA expression. However, there were no changes in the expression of *TrkA* or other NB differentiation markers (Figure 17B).
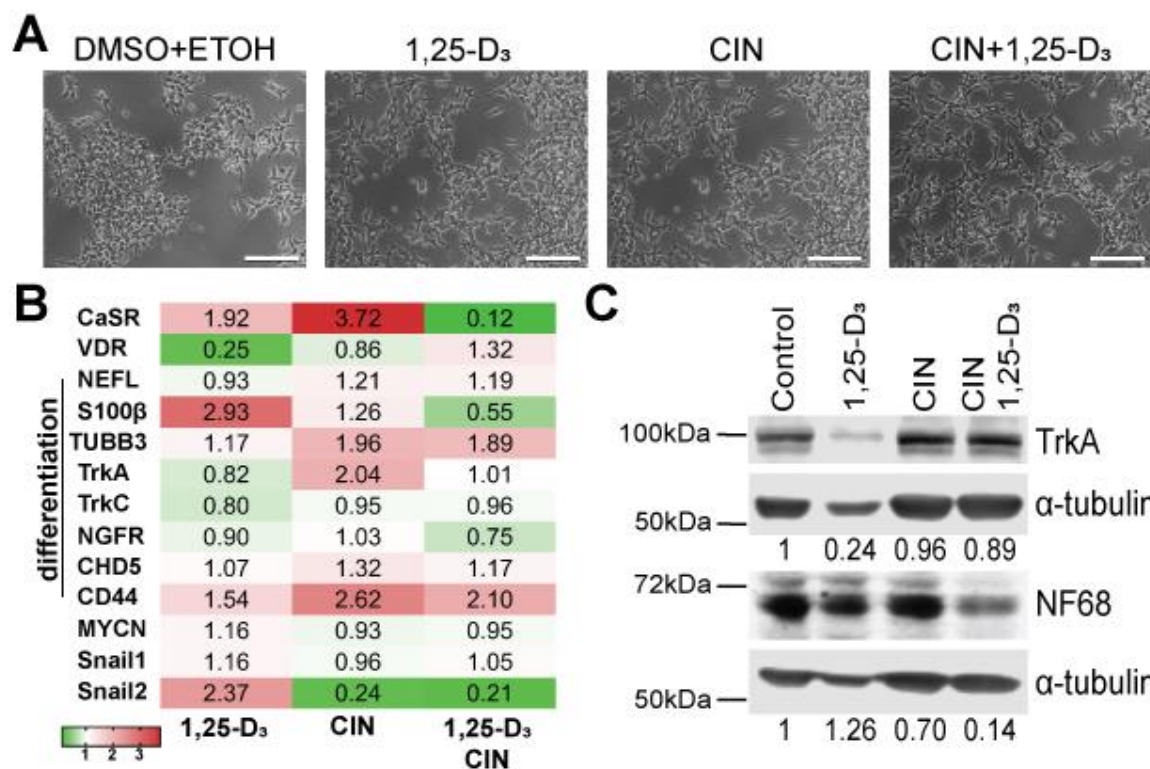


**Figure 17 – Effect of combination of 1,25-D$_3$ and CIN in LA-N-1 differentiation. (A)** Microphotography of LA-N-1 treated for 12 days with CIN or 1,25-D$_3$ alone or in combination or with their vehicle. White bar represents 1μm. **(B)** Total RNA was extracted from LA-N-1 treated for 12 days with either CIN or 1,25-D$_3$ alone or in combination. Heatmap of relative mRNA expression levels of specific genes determined by RT-qPCR and normalized relative to vehicle treated cells (DMSO+ETOH). **(C)** Expression of TrkA and NF68 analysed by immunoblots in cells treated for 12 days with CIN, 1,25-D$_3$ alone, in combination or their vehicle, Values under each blot represent quantification of each blot relative to α-tubulin and each treatment intensity was normalized to vehicle

Furthermore, we quantified protein levels of two NB differentiation markers, TrkA and NF68 (coded by *NEFL*). Single treatment of LA-N-1 with 1,25-D$_3$ decreased the protein levels of TrkA more than 70%, however treating cells with CIN alone or in combination with 1,25-D$_3$ had no effect on this NB marker. Treatment of LA-N-1 cells with CIN induced a 30% reduction of the protein levels of NF68, its combination with 1,25-D$_3$ reduced this protein to 14% of the DMSO treated cells (Figure 17C).

### 7.4 Combination of CIN and 1,25-D₃ increases expression of *CaSR* and NB differentiation marker in IMR5

In contrast to LA-N-1, IMR5 cells treated with both CIN and 1,25-D$_3$, showed longer dendrites than vehicle treated cells (DMSO+ETOH) suggesting a more differentiated phenotype. In addition, cells that received single treatment were morphologically similar to each other and to the control cells (Figure 18A). Analysis of the expression levels of *CaSR* showed that all treatment groups increased the expression, while the highest fold increase was induced by the combination of CIN and 1,25-D$_3$ (2-fold). Surprisingly, mRNA expression levels of genes associated with NB differentiation were reduced. Expression of *TrkC* and *NGFR* was 50% reduced when IMR5 were treated with 1,25-D$_3$ alone, and this was about 30%, when cells
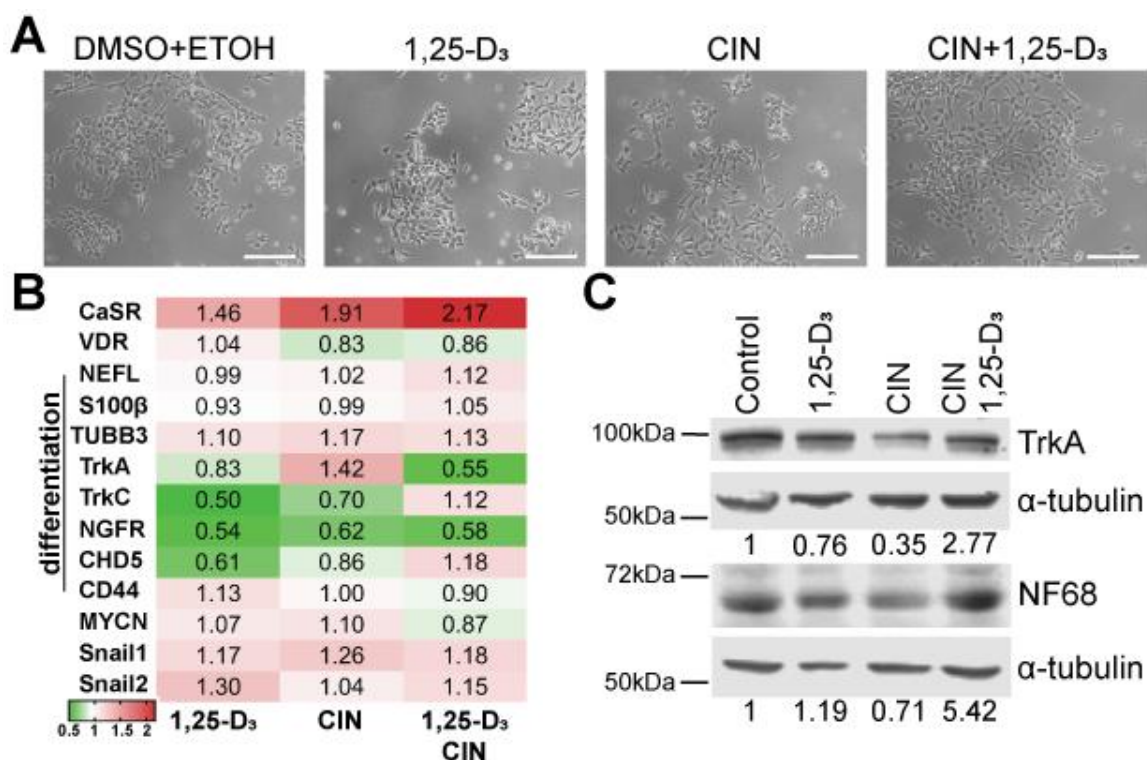


**Figure 18 – Combination of CIN and 1,25D$_3$ increases differentiation markers in IMR5**. **(A)** Microphotography of IMR5 that received CIN or 1,25-D$_3$ alone, in combination or their vehicle for 12 days. White bar represents 1µm. **(B)** IMR5 cells treated for 12 days with CIN and/or 1,25-D$_3$, or their vehicle were collected, and total RNA was extracted. Expression levels of the indicated genes was quantified by RT-qPCR and are relative to cells treated with vehicle (DMSO+ETOH). **(C)** Protein was extracted from IMR5 treated for 12 days with CIN, 1,25-D$_3$ alone, in combination or their vehicle. Immunoblots for TrkA and NF68 were done. Values under each blot represent quantification of each blot relative to tubulin and each treatment intensity was normalized to vehicle.

were treated with CIN. Treatment of IMR5 with the combination of 1,25-D$_3$ and CIN showed a 45% decrease of the mRNA expression of *NGFR* and *TrkA* (Figure 18B).

Concurring with the morphological changes observed, combination treatment increased NF68 and TrkA protein expression levels (more than 5- and 2- fold, respectively) suggesting a higher differentiation phenotype. However, treatment of IMR5 with CIN alone resulted in a reduction of the amount of TrkA protein level expression (Figure 18C).

### 7.5 NB cell lines have a differential expression of VDR

To further understand the different responses obtained when IMR5 and LA-N-1 cell lines were treated with 1,25-D$_3$, alone or in combination with CIN. To do this we studied both the mRNA and protein levels of VDR in the NB cells lines available in the Laboratory of Developmental Tumours of the HSJD. Analysis of the mRNA levels showed a differential pattern of expression, while LA-N-1 are the NB cell lines with the lowest expression of *VDR*, IMR5 have 15-fold more mRNA expression (Figure 19A). Accordingly, LA-N-1 cells also showed the lowest protein expression of VDR, while the VDR band of IMR5 was 60 times more intense (Figure 13B). Of not note, the cell line that presented the highest expression of *VDR* mRNA was the SK-N-SH, while the highest VDR protein expression was found in the SK-N-AS (Figure 19A).
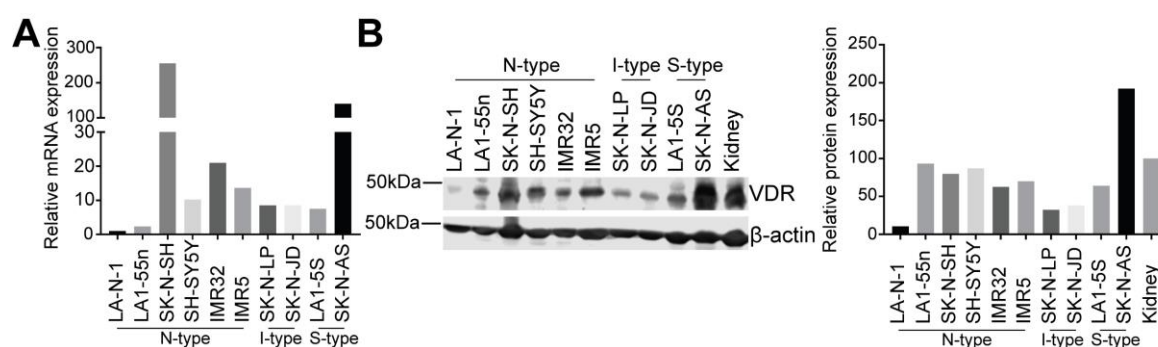


**Figure 19 − Expression of VDR in NB cell lines. (A)** Relative mRNA expression levels of *VDR* in 10 NB cell lines measured by RT-qPCR and normalized to LA-N-1. **(B)** Immunoblot showing VDR protein expression levels in NB cell lines. Human kidney protein extracts were used as a control. Band intensities were quantified relative to β-actin. Bars represent fold change relative to LA-N-1

# 8  Combination of Retinoids and CIN in the treatment of neuroblastoma

Retinoids play an important role in the differentiation of tissues (*Niederreither et Dolle – 2008*) and currently, they are administrated after consolidation therapy for high-risk NB patients (*Whittle* et al – *2017*). CaSR is expressed in benign, more differentiated NBs (*de Torres* et al – *2009*) and our group has previously shown that ATRA can increase the expression of this GPCR in NB cell lines (*Casalà* et al – *2012*). Thus, we hypothesised that retinoids might potentiate the effect of CIN in NB cell lines by upregulating the expression of CaSR. To test this, we used two forms of retinoic acid: ATRA, the most common and studied retinoid, and 13-*cis*-RA, a retinoid used in the treatment of NB during the maintenance phase (*Whittle* et al – *2017*).

## 8.1  Retinoids increase CaSR levels in NB cell lines

To validate our hypothesis, we first evaluated the effect of retinoids in *CaSR* mRNA expression We treated two NB cell lines that exhibited a low endogenous expression of this receptor with two different retinoids (ATRA and 13-*cis*-RA) and evaluated *CaSR* mRNA and protein expression levels.

Although high variability between assays was observed, during the first hours of the treatment, *CaSR* mRNA expression was increased in LA-N-1 cells treated with ATRA (2- and 4- fold) (Figure 20A, top panel). Similarly, IMR5 treated cells increased the expression of this receptor (more than 2-fold) in two and three of the assays performed, respectively (Figure 20A, bottom panel). Next, we evaluated the effect that ATRA had in the CaSR protein levels of LA-N-1 and IMR5 after treatment. In both cell lines two bands were detected, one at approximately 100kDa which corresponds to the non-glycosylated immature CaSR, and another at around 130kDa, corresponding to the mature CaSR monomer. However, there was no visible band at 240kDa, which corresponds to the CaSR dimer. Immunoblot quantification showed that LA-N-1 treated with ATRA for 8- or 24-hours induced a decrease of CaSR monomer levels, while they showed a 2-fold increase of the non-glycosylated bands. Nevertheless, cells treated with ATRA for 48-hours presented a 40% reduction of both forms of CaSR protein (Figure 20B, top panel).  In IMR5 cells,

this retinoid induced a slightly increase of both CaSR monomer and non-glycosylated bands, after 48 hours of treatment. (Figure 20B, bottom panel).
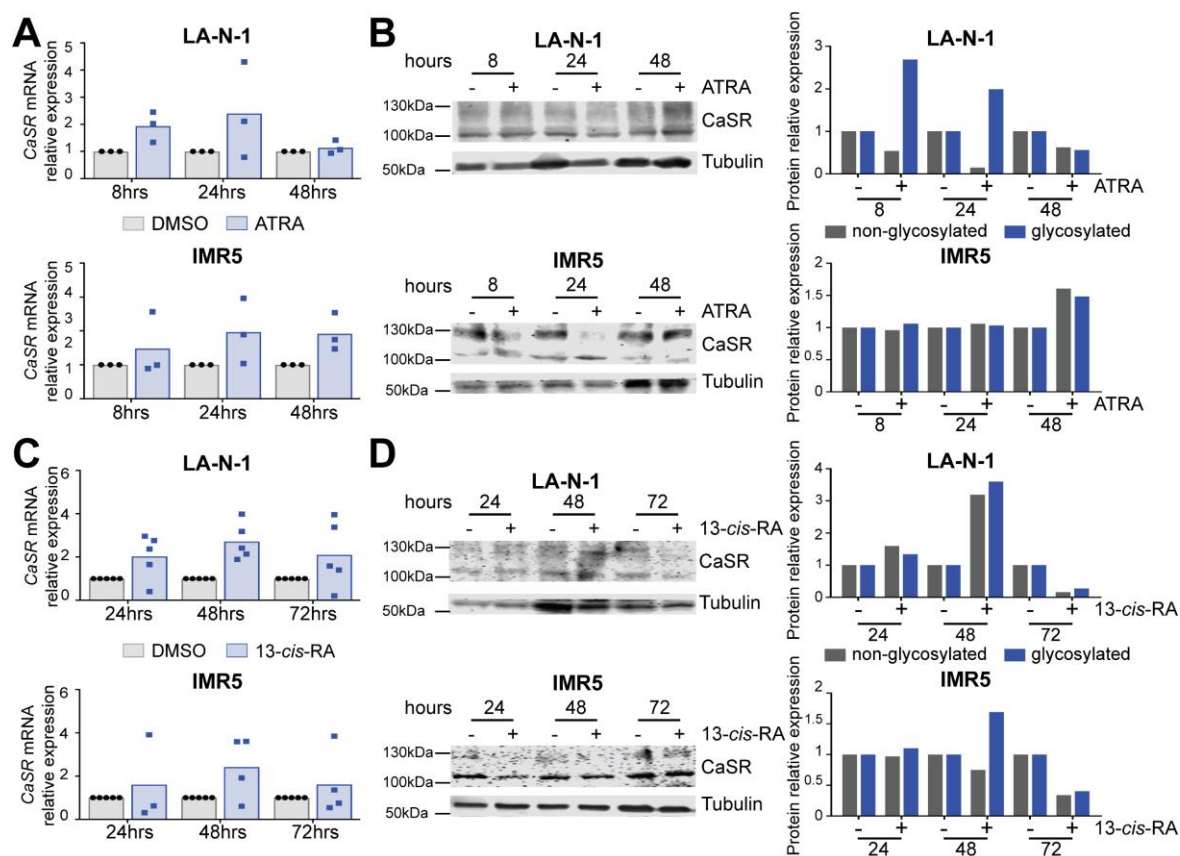


**Figure 20 – Effect of retinoids in CaSR expression in NB cell lines. A**– LA-N-1 and IMR5 cells were treated with 2.5µM ATRA or DMSO for the indicated times. *CaSR* mRNA was quantified by qPCR, using TBP as reference gene. Bars represent means and points individual experiments. **B-** CaSR immunoblot of LA-N-1 and IMR5 treated with 2.5µM ATRA for the indicated times. Band intensity was quantified relative to tubulin and normalised to DMSO control at each timepoint. **C-** Quantification of CaSR mRNA expression of LA-N-1 and IMR5 cells treated with 2.5µM 13-*cis*-RA or DMSO for the indicated times. RT-qPCR was used to detect *CaSR* mRNA and quantification was done by using TBP as reference gene. Bars represent means and points individual experiments. **D-** CaSR immunoblot of LA-N-1 and IMR5 treated with 2.5µM ATRA for the indicated times.

13-*cis*-RA is used in the treatment of several oncological diseases due to the more favourable pharmacokinetics, when compared to ATRA. This retinoic acid acts as a pro-drug since it is isomerized to ATRA intracellularly (*Armstrong* et al – *2005*). Due to its longer mechanism of action, we evaluated the effect of this drug had on CaSR expression levels. Despite the high variability between assays, LA-N-1 cells treated with 13-*cis*-RA for 24 hours increased their *CaSR* mRNA expression more than 2-fold relative to DMSO treated cells. Moreover, the increase of expression was

greater when cells were exposed for prolonged periods of time (4-fold increase after 48 and 72 hours of treatment) (Figure 20C, top panel). Like LA-N-1 cells, IMR5 cells showed high variability in *CaSR* mRNA levels due to 13-*cis*-RA exposure resulting in an up-regulation of more than 2-fold when cells were exposed for prolonged times. (Figure 20C, bottom panel). Additionally, we evaluated the effect that 13-*cis*-RA presented in CaSR protein levels of NB cell lines. Quantification of immunoblots showed that treatment of LA-N-1 cells for 24-hours with 13-*cis*-RA increased the expression of mature CaSR 1.5-fold, a 48-hour treatment increased its expression by 3-fold. Effect of 13-*cis*-RA in immature non-glycosylated CaSR of LA-N-1 was similar. IMR5 cells treated with 13-*cis*-RA showed 1.6-fold increase of immature CaSR after 48-hours treatment, and a 3-fold reduction of the mature CaSR after 72-hours of treatment. Although quantification of the immunoblots was feasible, the deficient quality of the imaging could have led to an over or underestimation of the results (Figure 20D). Taken together, our findings showed an increase of CaSR mRNA and protein levels after 24-hours of treatment with ATRA and after 48 hours of treatment with 13-*cis*-RA.

## 8.2   Retinoids reduce cell viability and colony formation capacity of NB cell lines

We aimed at exploring the effect of CIN on NB cell viability when combined with a retinoid, either ATRA or 13-*cis*-RA.  After 2 days of treatment with the combination of ATRA and CIN, cell viability of LA-N-1 was lower than cells treated with DMSO (day 2, DMSO vs ATRA+CIN p=0.0020). However, viability of these cells was not significantly different from cells treated with ATRA alone (Figure 21A, top panel). Treating IMR5 with ATRA reduced cell viability at experimental day 2 ((p=0.0017, ATRA vs DMSO). Moreover, this effect was diminished in the consecutive experimental days and there was no statistical significance in the viability of cells treated with ATRA or DMSO. Combination of ATRA and CIN significantly reduces IMR5 viability after 2 (p=0.0225), 3 (p=0.0197) and 4 (p=0.0076) days of treatment, compared to DMSO (Figure 21A, bottom panel).
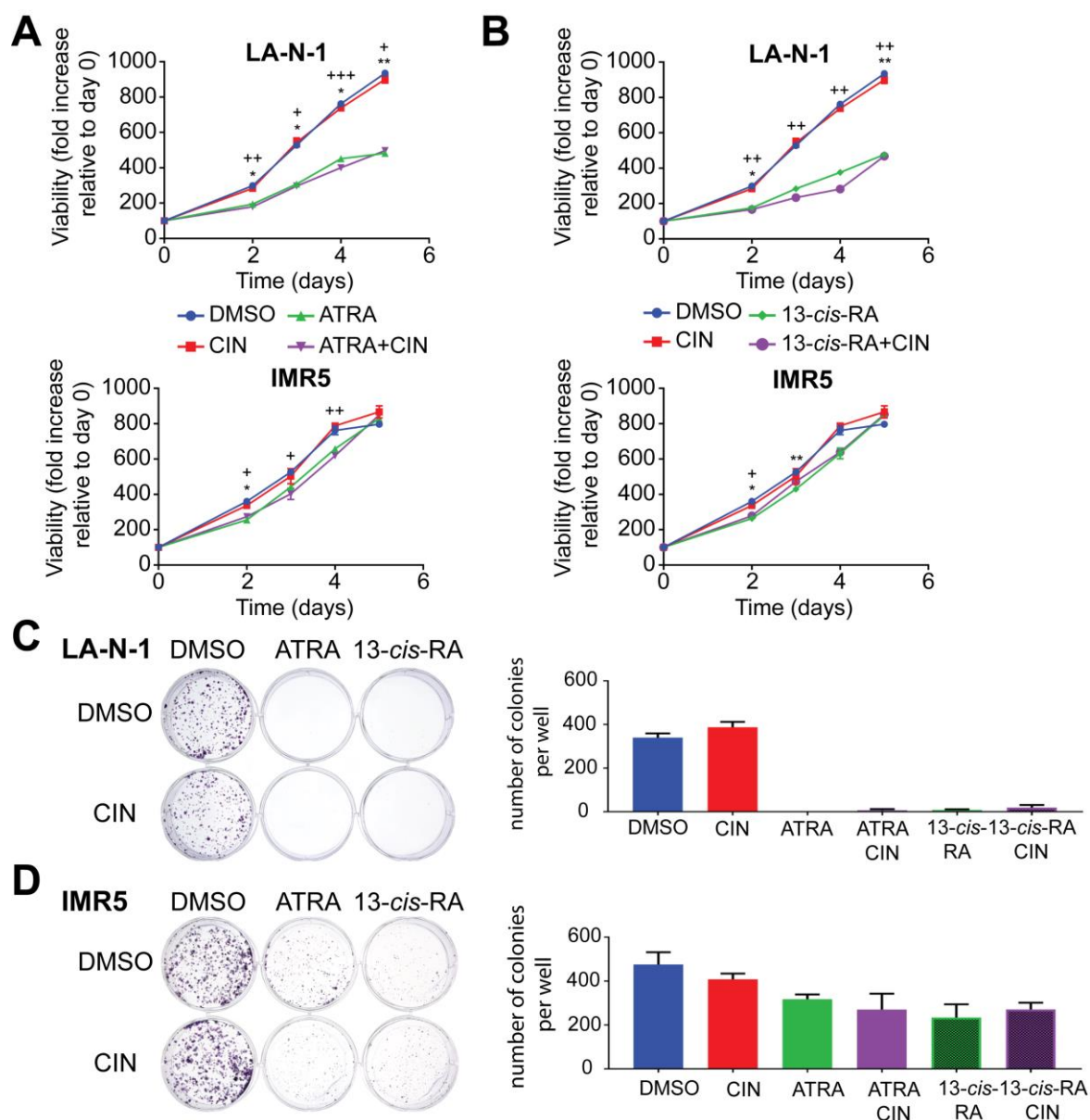
**Figure 21 – NB cell viability is reduced by retinoids treatment – A, B** LA-N-1 or IMR5 were treated with 2.5µM of ATRA or 2.5µM of 13-*cis*-RA for 24 hours before 1µM of CIN was added. Cell viability was measured by MTS, 2, 3, 4 or 5 days after adding CIN, as each point represents media±SEM of six technical replicates. Statistical significance was calculated at each day using one-way ANOVA with Dunn's post correction. *$p<0.05$ **$p<0.01$ ATRA vs DMSO +$p<0.05$ ++$p<0.01$ +++$p<0.001$ ATRA+CIN versus DMSO **C,D** LA-N-1 (1000 cells) or IMR5 (2500 cells) were seeded in 6-well plates and treated for 24hours with 2.5µM of ATRA or 13-*cis*-RA before adding 1µM of CIN. Cells were fixed, stained and photographed after 12 days of treatment. Number of colonies in each well was counted using STRATA Quest from three technical replicates.

Next, we evaluated the effect that 13-*cis*-RA and its combination with CIN had on viability of NB cell lines. After 2 days of treatment, viability of LA-N-1 cells treated with 13-*cis*-RA alone or in combination with CIN was significantly lower than

DMSO treated cells (p=0.0423 and p=0.0020, respectively). At day 3 and 4, combined treatment significantly reduced cell viability (day 3, p=0.0042, day 4, p=0.004), while the effect of 13-*cis*-RA alone was not statistically significant (day 3, p=0.1485; day 4, p=0.0539) compared to DMSO treated cells. Throughout the assay, viability of LA-N-1 treated with CIN was similar to cells treated with DMSO (Figure 21B, top panel). Effect of 13-*cis*-RA single treatment was not as expressive in IMR5 as in LA-N-1. However, there was a significant reduction of cell viability when compared to DMSO treated cells (day 2, p=0.0018, day 3, p=0.0087). After 2 days of combined treatment, IMR5 viability was significantly reduced (p=0.0184) when compared to DMSO treated cells. However, this difference was not statistically significant in the other timepoints. In addition, IMR5 viability was not affected when cells were treated with CIN alone compared to the DMSO treated control cells (Figure 21B, bottom panel).

Due to experimental limitations, viability assays cannot be maintained for more than 6 days, as control cells get too confluent and start dying. Therefore, we evaluated the effect that retinoids and their combination with CIN had on the colony formation capacity of NB cell lines. Treatment of LA-N-1 with ATRA or 13-*cis*-RA, totally abrogated the ability of this cell lines to form colonies, there were no visible colonies. Effect of these retinoids alone was so strong that its combination with CIN was insignificant. LA-N-1 cells treated with CIN alone had 14% more colonies than DMSO treated cells, however this was not statistically significant (Figure 21C). Retinoids treatment was not as effective in reducing colony formation capacity of IMR5 as in LA-N-1. However, size and number of IMR5 colonies in wells treated with retinoids were remarkably lower than in DMSO or CIN treated wells. Quantification of the number of colonies showed 33% decrease in cells treated with ATRA alone and this effect was increased to 43% when combined with CIN. For IMR5 cells, the colony formation capacity was reduced by 50% when cells were treated with 13-*cis*-RA, while its combination with CIN only reduced number of colonies by 40% (Figure 21D).

## 8.3   Retinoids receptors are differently expressed in NB cell lines

To understand the differential response that LA-N-1 and IMR5 showed to retinoids, three retinoid receptors expression levels were assessed in NB cell lines: retinoid acid receptor alpha (RARα) retinoid acid receptor gamma (RARγ) and retinoid X receptor alpha (RXRα) (*Bushue* et *Wan – 2010*). All of them exhibited endogenous expression, although the highest mRNA expression was detected in SK-N-AS cells and the lowest expression in SK-N-LP.  When we compared the expression of these receptors in LA-N-1 and IMR5 cells, the latter expressed six times more *RARγ*. However, the expression of *RXRα* was reduced approximately 50% relative to LA-N-1 (Figure 22A). In contrast, analysis of protein expression showed that IMR5 was the cell line with the highest protein levels of RARα, having twice the amount observed in LA-N-1. In addition, LA-N-1 cells had the highest
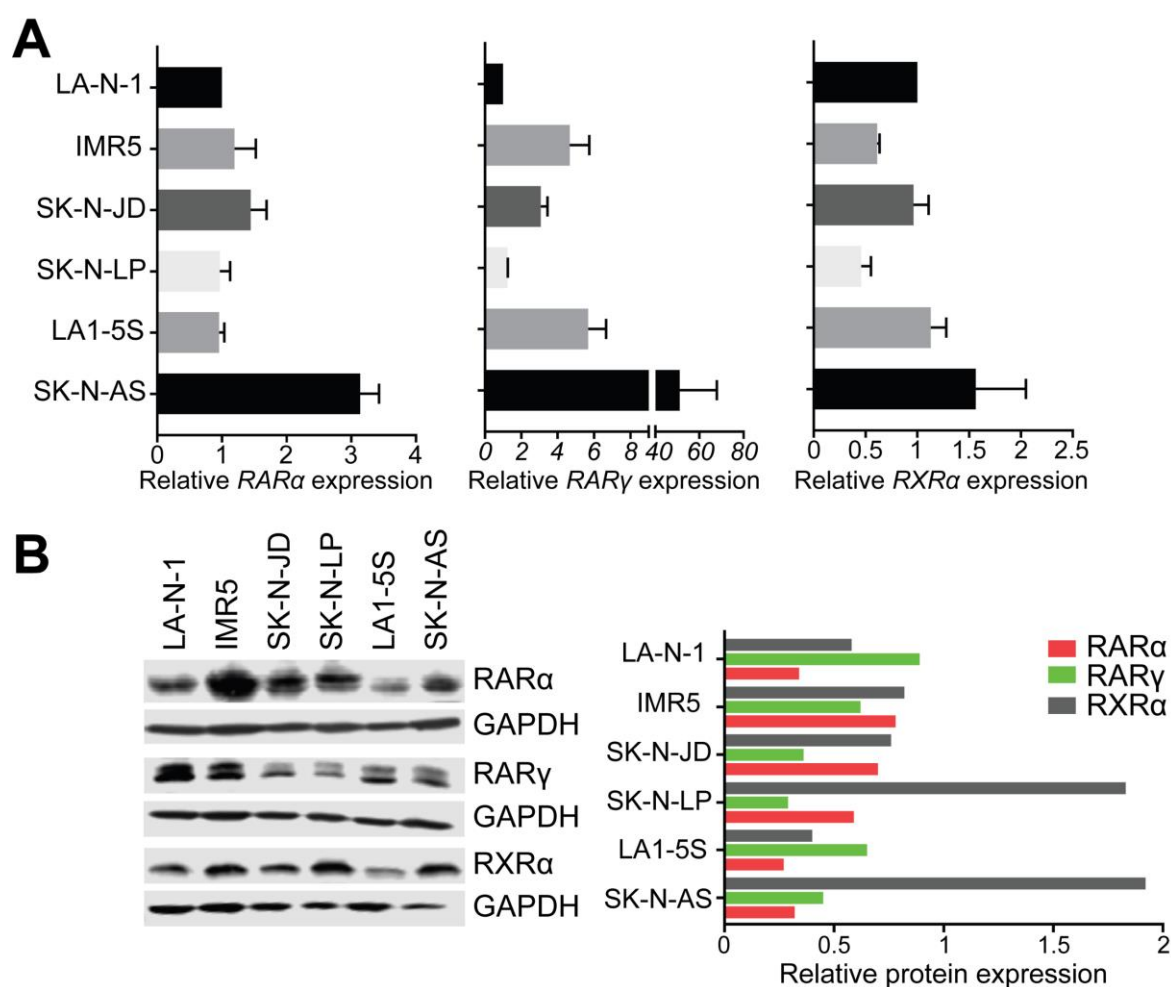


**Figure 22 – Expression of retinoid receptors in NB cell lines**. (A) Relative mRNA expression of RARα, RARβ and RXRα in 6 different NB cell lines measured by RT-qPCR and normalized to LA-N-1. (B) Immunoblot of RARα, RARβ and RXRα of six NB cell lines. Band intensity was quantified relative to GAPDH for each receptor.

protein levels of RARγ while IMR5 presented 30% less. SK-N-AS cells had the highest protein expression of RXRα, IMR5 cells had twice the amount of this receptor than LA-N-1 cells (Figure 22B).

## 8.4 ATRA and 13-*cis*-RA have a differential effect on NB differentiation

Retinoids play a pivotal role in cell differentiation during embryogenesis, and it is this property that makes them interesting therapeutical drugs in cancer treatment (*Niederreither* et *Dolle* – 2008). Thus, we evaluated the effect that ATRA and 13-*cis*-RA alone or in combination with CIN had on differentiation of NB cell lines. LA-N-1 cells treated with ATRA or 13-*cis*-RA alone showed an enlargement of cells and longer dendrites suggesting a more differentiated phenotype. The biggest difference between treated cells was the morphological appearance of the colonies, LA-N-1 treated with ATRA formed compact aggregates of overlapping cells, whereas cells treated with 13-*cis*-RA grew in flatter, spread out colonies. Treatment with retinoids in combination with CIN induced a differentiated morphology as well as an increase
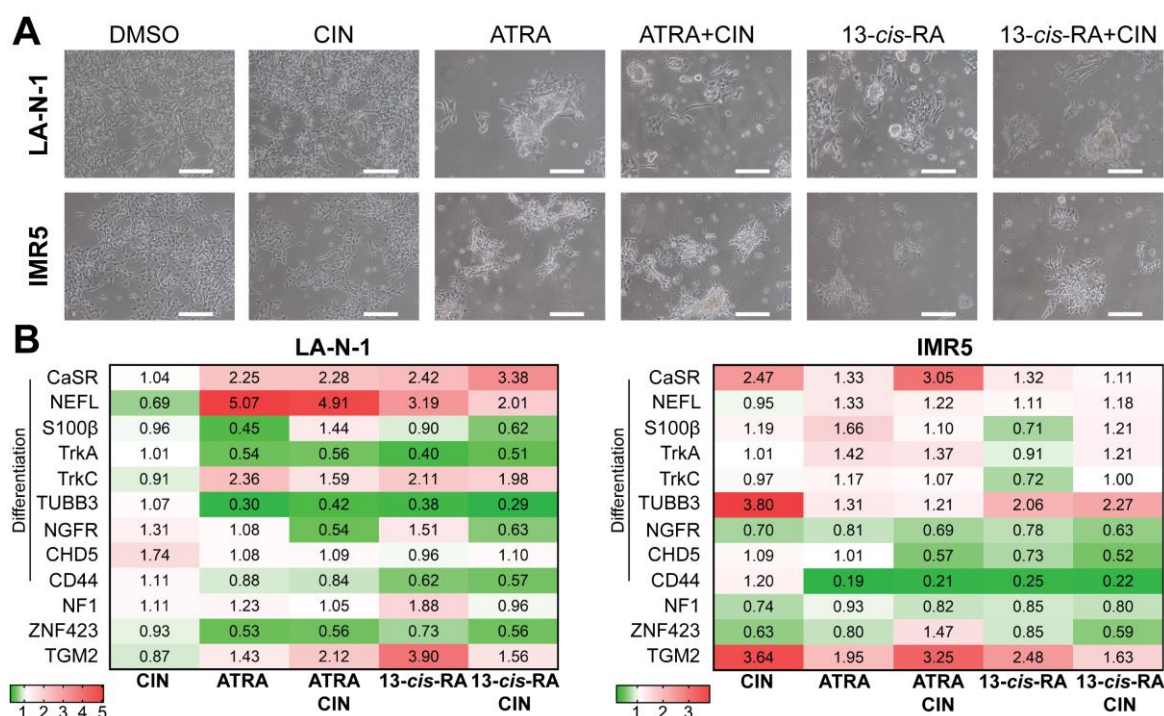


**Figure 23 – Retinoids induce differentiation of NB cell lines – (A)** Microphotography of LA-N-1 and IMR5 treated for 12 days with 2.5μM of ATRA or 13-*cis*-RA alone or in combination with 1μM CIN or DMSO. **(B)** LA-N-1 and IMR5 cells were treated for 12 days with 2.5μM of ATRA or 13-cis-RA alone or in combination with 1μM CIN or DMSO and mRNA levels of the indicated genes were quantified by RT-qPCR. Values indicate fold change relative to DMSO of three biological replicates.

of floating apoptotic bodies, while, cells treated with CIN were morphologically similar to DMSO treated cells (Figure 23A, right panel). IMR5 treated with CIN appeared to be more differentiated than control cells treated with DMSO, with further dendrites and a more spread-out morphology. IMR5 cells treated with ATRA or 13-*cis*-RA formed sparse aggregates of cells with long interconnected processes. Combination of CIN with either retinoid did not increase the differentiation of IMR5 cells induced by either agent alone (Figure 23A, left panel).

In addition to morphological changes we also evaluated the expression of several genes associated with NB differentiation, retinoic acid response and resistance. *CaSR* mRNA expression showed more than 2-fold increase in LA-N-1 cells treated with ATRA or 13-*cis*-RA. However, this effect was not increased by their combination with CIN. In LA-N-1 cells NB differentiation associated genes were modified differently by each treatment. After 14 days of treatment with CIN, LA-N-1 cells increased by 1.7-fold mRNA levels of *CHD5*. Single treatment with ATRA increased by 5-fold the levels of *NEFL* and 2.36-fold expression of *TrkC*, similarly 13-*cis*-RA single treatment increased 3- and 2.18-fold these two genes, this effect was not altered when CIN was added to the treatment. Expression of *TrkA* and *TUBB3* was reduced by retinoid treatment (2 and 3-fold respectively), and combination with CIN did not alter this effect. Expression of *NGFR* was 2-fold less when cells were treated with combination of CIN and retinoids (either ATRA or 13-*cis*-RA), however retinoid single treatment did not affect its expression. Another gene that was differentially expressed when retinoids were combined with CIN was *S100β*, its expression was reduced upon ATRA single treatment and increased when cells are treated with ATRA+CIN (Figure 23B, right panel).

Next, we evaluated the expression of *NF1* and *ZNF423,* genes involved in retinoid resistance (*Hölzel* et al – *2010*). Although *NF1* expression was not modified by any treatment in LA-N-1 cells, *ZNF423* expression was downregulated after retinoid treatment. However, this effect was not increased by their combination with CIN. Additionally, treatment with 13-*cis*-RA or ATRA alone or in combination with CIN, induced an upregulation of *TGM2,* a target gene of retinoic acid receptor (Figure 23B, right panel).

IMR5 cells treated for 14 days with a single treatment of CIN increased more than 2-fold their *CaSR* mRNA levels. Combination of CIN and ATRA induced a 3-fold increment of the expression of this GPCR, however single treatment with either

retinoid maintain de levels of *CaSR*. *TUBB3* showed the most significant changes, 13-*cis*-RA alone or in combination increase its expression by 2-fold and single treatment induced a 3-fold increment in mRNA levels. In the treatment groups where retinoids were present there was a 5-fold decrease of CD44 expression, whereas combination of retinoids with CIN had a similar effect as single treatment (Figure 23 B, left panel).

Genes associated with retinoid resistance, *NF1* and *ZNF423*, were not altered by any treatment. Expression of the retinoid response gene, *TGM2*, was increased 3-fold in IMR5 cells treated with CIN alone or in combination with ATRA, and 2-fold with ATRA single treatment. Additionally, 13-*cis*-RA alone increased 2.6-fold expression of *TGM2* while its combination with CIN lead to a 1.6-fold increase (Figure 23B left panel).

### 8.5  *In vivo* exposure to ATRA does not increase CaSR expression in NB xenograft

Considering our *in vitro* results, we hypothesized that a pre-treatment of NB xenograft models with ATRA could increase the efficiency of CIN. Thus, we treated LA-N-1 xenograft models daily with an intraperitoneal (IP) injection of ATRA (2.5mg/kg/day) for 1, 2 and 3 weeks. After one week of treatment, tumour volume was not affected as compared to vehicle treated tumours. ATRA treated tumours had a 47% smaller volume than VEH after 2 weeks of treatment (p=0.0736), while after 3 weeks the difference in volume between ATRA and VEH treated tumours was negligible (p=0.7619) (Figure 24A).

Our main interest with this experiment was to evaluate if ATRA could increase CaSR expression *in vivo*. Using RT-qPCR we quantified the mRNA expression of this GPCR, *NEFL* and *TrkA,* two NB differentiation markers. Although we were unable to detect *CaSR* mRNA in all treated tumours, *CaSR* mRNA levels are near the detection range, we observed that ATRA did not increase *CaSR* expression of xenograft models. Moreover, mRNA expression of *NEFL* and *TrkC* was not affected by this retinoid (Figure 24B, left panel). Consistent with these results, histological analysis of tumours treated with ATRA or vehicle showed no morphological differences in tumour cells. In addition, evaluation of CaSR protein expression by IHC showed no differences between the tumours that received ATRA or VEH (Figure 24C).
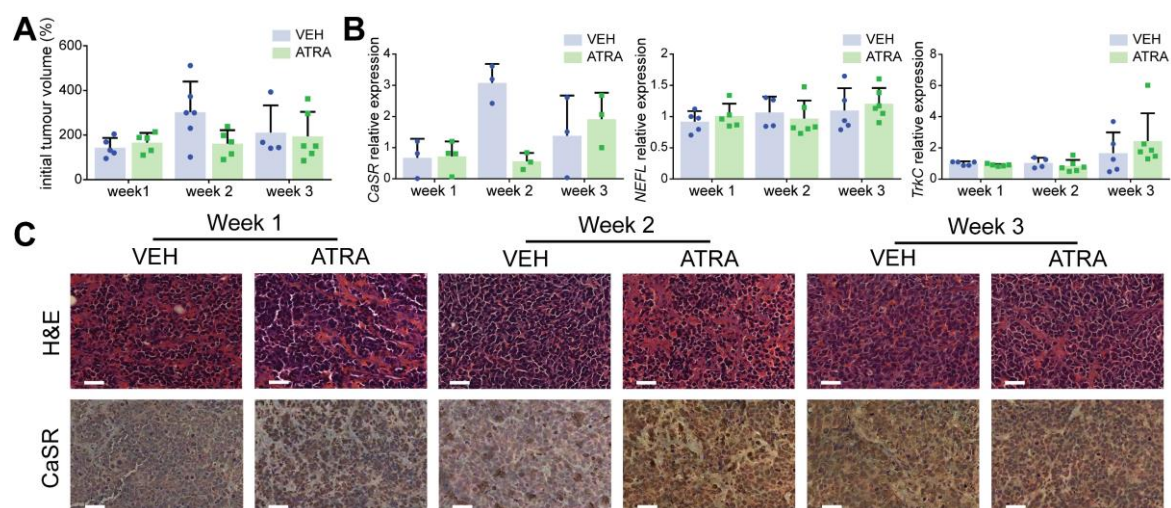
**Figure 24 – Exposure of NB xenografts to ATRA does not increase CaSR expression.** LA-N-1 cells were injected subcutaneously into each flank of four to six-old athymic nude-Foxn 1 *nu/nu* mice. When tumours reached a volume between 150-200mm$^3$ mice were treated daily with 2.5mg/kg of ATRA or its vehicle (VEH) intraperitoneally. Tumours were collected after 1, 2 or 3 weeks of treatment. **(A)** Variation of tumour volume was calculated for each tumour and was represented as percentage of initial volume. Bars represent mean ± SEM and each point represent an individual tumour. **(B)** Relative mRNA expression of *CaSR*, *NEFL* and *TrkA* from LA-N-1 xenograft tumours treated for 1, 2 or 3 weeks with ATRA or VEH, measured by RT-qPCR and normalized to LA-N-1. Bars represent mean ± SEM and each point represent an individual tumour. **(C)** H&E and CaSR IHC of formalin fixed, paraffin-embedded section of LA-N-1 tumours treated with ATRA or VEH for 1, 2 or 3 weeks, white bar represents 40μm.

## 8.6 EFS of LA-N-1 xenografts is increased by daily treatment with ATRA combined with CIN

CaSR levels in LA-N-1 xenograft models treated with ATRA for 1, 2 or 3 weeks were not increased, thus, we decided to evaluate the effect that combination of CIN and ATRA had on LA-N-1 xenografts without pre-treatment with ATRA. We treated mice daily with an oral dose of 10mg/kg of CIN alone or in combination with a daily IP dose of 2.5mg/kg of ATRA. Vehicle treated mice received both, an oral dose of vehicle of CIN and an IP dose of vehicle of ATRA. Animals that received ATRA or its vehicle started to develop an abdominal swelling after 7 weeks of treatment, suggesting this was a result of the accumulation of ATRA's vehicle due to its low absorption. Because of this, several tumours never reached the end threshold volume of 1500mm$^3$, as animals were sacrificed earlier for ethical reasons. Thus, we analysed EFS Kaplan-Meier curves of the four treatment groups considering the event as "tumour reaching 1000mm$^3$". Treatment with ATRA alone or in combination

with CIN significantly inhibited tumour growth (p=0.0048 and p=0.0006, respectively). As we previously reported, CIN needed prolonged exposure to inhibit tumour growth.
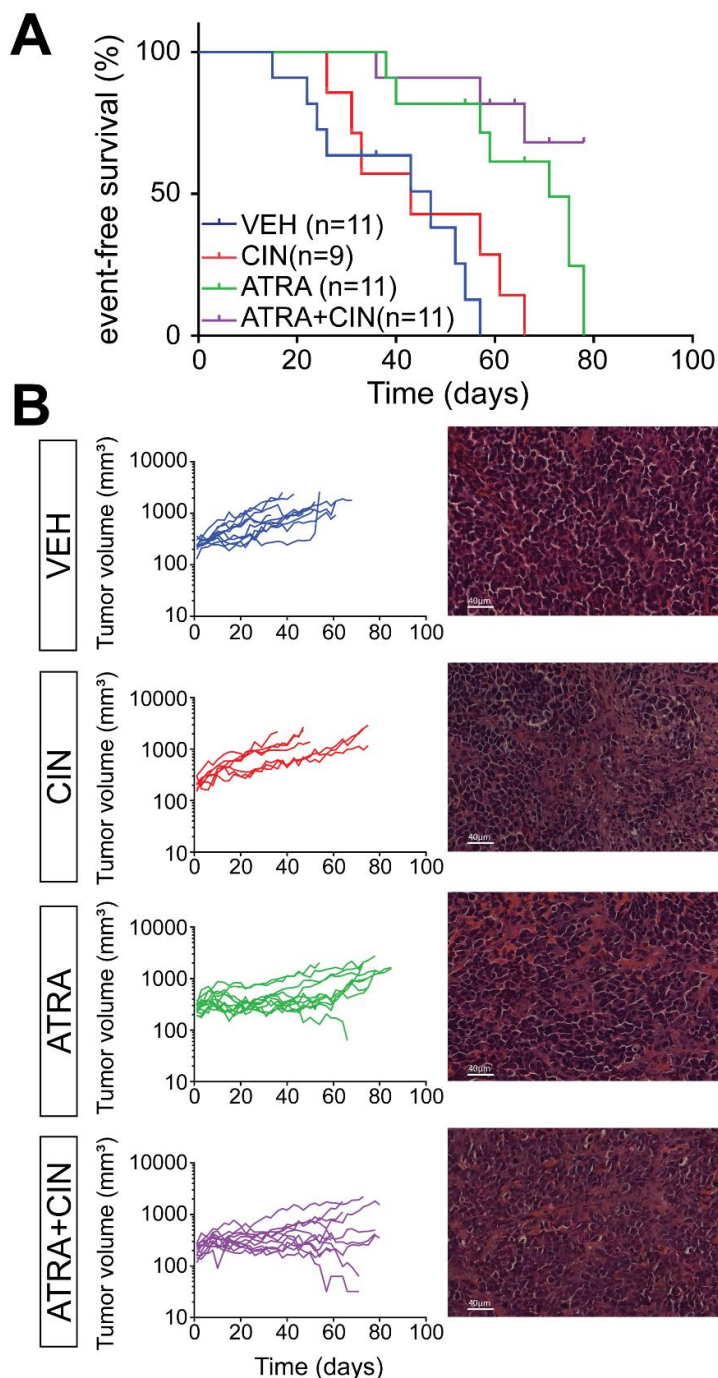


**Figure 25 – Daily treatment with ATRA combined with CIN inhibits NB tumour growth**. LA-N-1 cells were injected subcutaneously into each flank of four to six-old athymic nude-Foxn 1 *nu/nu* mice. When tumours reached a volume between 150-200mm$^3$ mice they were randomly assigned to each treatment group and treated daily with 2.5mg/kg of ATRA (intraperitoneally), 10mg/kg CIN (orally), alone or in combination or with their vehicle (VEH). Animals were treated until tumours reached 2000mm$^3$ or after 10 weeks of treatment. **(A)** Kaplan-Meier event-free survival curves of mice treated with ATRA, CIN alone or in combination and with their vehicle. Event was considered reaching 1000mm$^3$, and log-rank test with a Bonferroni correction was used to compare the survival curves between groups (VEH vs ATRA p=0.0048; VEH vs ATRA+CIN p=0.0006; ATRA vs ATRA+CIN vs CIN p=0.00054; ATRA vs CIN p=0.0432). **(B)** Representation of individual tumour growth in each treatment group. H&E of formalin fixed, paraffin-embedded section of LA-N-1 tumours treated with ATRA, CIN alone or in combination with their VEH, white bar represents 40µm.

Kaplan-Meier survival curves of tumours treated with CIN or vehicle were not significantly different (p=0.156). EFS curves of tumours treated with ATRA or its combination with CIN were significantly different from EFS from tumours treated with CIN (p=0.0432 and p=0.0054, respectively) (Figure 25A). Furthermore, there was one tumour treated with ATRA and two tumours treated with the combination with ATRA and CIN that had a volume reduction to a non-palpable mass (Figure 25B, right panel). Histological evaluation of tumours treated with CIN showed cells with a slightly larger cytoplasm than the VEH treated tumours. Similarly, tumours treated with ATRA and the combination showed cells with large cytoplasm as well as large fibrotic areas (Figure 25B, left panel), suggesting a more differentiated status.

## 8.7 Combination of ATRA and CIN have antagonistic effect in NB tumour growth

Due the toxicity of ATRA's vehicle we decided to test the effect of different posology of ATRA. We administered ATRA once and three times per week (ATRA (1d) or ATRA (3d), respectively) (2.5mg/kg/day) alone or in combination with CIN. CIN and ATRA (3d) as single treatment, significantly inhibited tumour growth (p=0.0048 and p=0.0024, respectively). However, combination of CIN with ATRA (1d or 3d) did not inhibit tumour growth, in fact, mean survival of the groups ATRA+CIN (1d) and ATRA+CIN (3d) was 20.5 days and 31 days, respectively, while mean survival of vehicle treated tumours was 20 days (Figure 26A).

Analysis of the behaviour of single treatments, revealed that tumours treated with CIN grew slower than tumours treated with VEH. All tumours that received ATRA three times a week were treated for at least 8 weeks before reaching an end-point volume, three of these tumours were in treatment for 10 weeks, experimental end-point, did not reach a volume of 1000mm$^3$. Tumours treated with ATRA once a week grew faster than the group ATRA (3d). However, there were two tumours that regressed to a non-palpable mass after 6 weeks of treatment. Animals bearing these tumours were treated up to experimental endpoint (10 weeks) and tumours never reappeared. Tumours treated with ATRA once or three times a week in combination with CIN, showed growth curves similar to VEH treated tumours (Figure 26B).

We also analysed histological features of tumours from these six treatment groups. Tumours treated with CIN showed cells with a bigger cytoplasm and presented more necrotic areas than VEH treated tumours. Cells from tumours

treated with ATRA once a week had a similar morphology as cells from tumours treated with CIN alone. However, tumours treated with ATRA three times per week had more necrotic and fibrotic areas than tumour treated with VEH, ATRA (1d) or CIN.
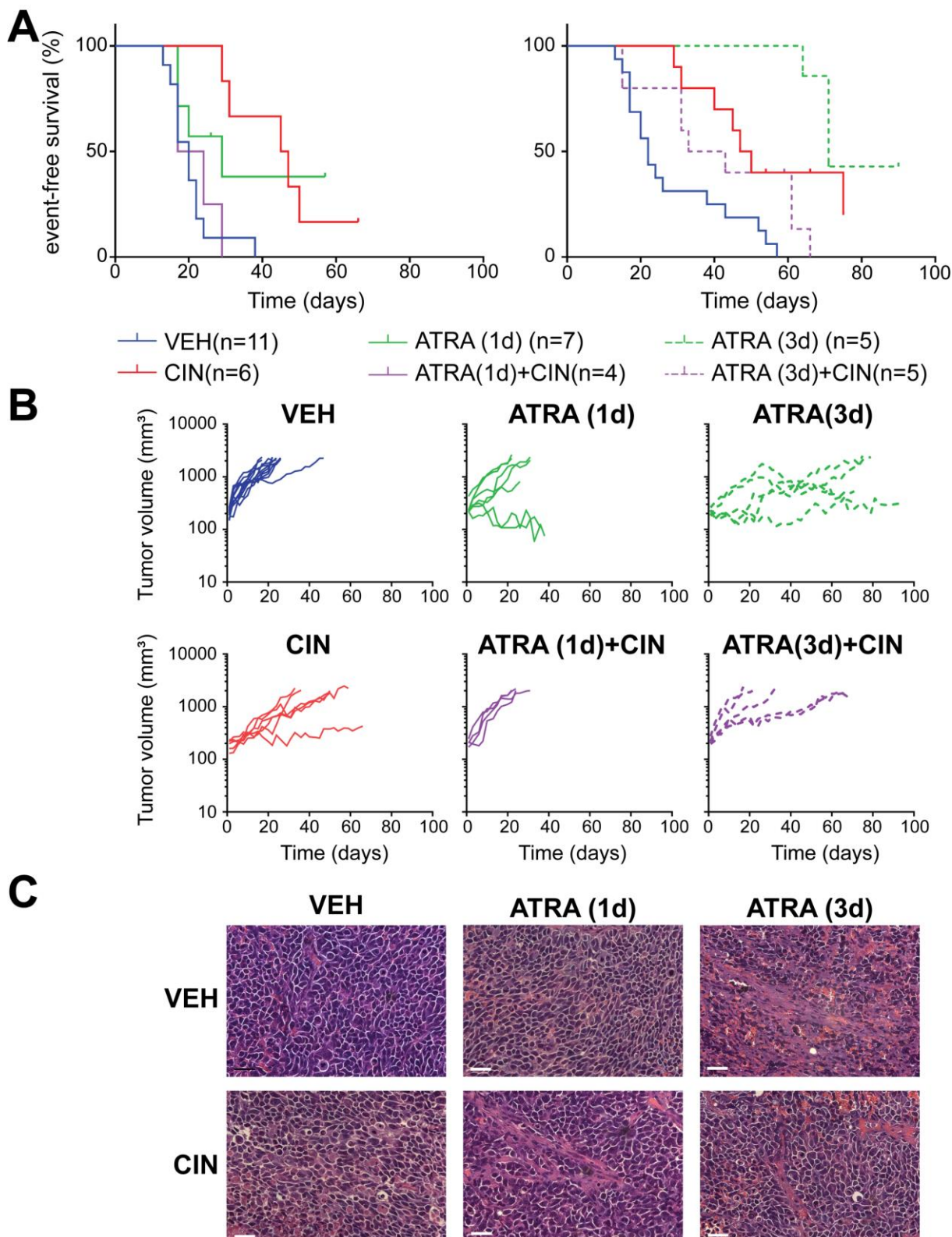
**Figure 26 – Combination of ATRA and CIN have an antagonistic effect in NB tumour growth.** LA-N-1 cells were injected subcutaneously into each flank of four to six-old athymic nude-Foxn 1 *nu/nu* mice. When tumours reached a volume between 150-200mm$^3$ mice they were randomly assigned to each treatment group and treated one (1d) or three (3d) times per week with 2.5mg/kg of ATRA (intraperitoneally) alone or in combination with a daily dose of 10mg/kg CIN (orally), alternatively animals received vehicle (VEH). Animals were treated until tumours reached 2000mm$^3$ or after 10 weeks of treatment. **(A)** Kaplan-Meier event-free survival curves of mice treated with once (1d) or three (3d) times a week ATRA alone or in combination with CIN or their vehicle. Event was considered reaching 1500mm$^3$, and log-rank test with Bonferroni correction was used to compare the survival curves between groups (VEH vs CIN p=0.0048; VEH vs ATRA(3d) p=0.0024; ATRA (3d) vs ATRA(3d)+CIN p=0.0396; CIN vs ATRA(1d)+CIN p=0.0192). **(B)** Representation of individual tumour growth in each treatment group. **(C)** H&E IHC of formalin fixed, paraffin-embedded section of LA-N-1 tumours treated with ATRA(1d) or ATRA(3d), CIN alone or in combination with their VEH, white bar represents 40µm.

## 8.8 *In vivo* combination of CIN and 13-*cis*-RA impacts NB tumour growth

ATRA is the most common retinoid used for research purposes. However, due a more favourable pharmacokinetics, NB patients are treated with oral 13-*cis*-RA administered during the maintenance treatment phase (*Whittle* et al – *2017*). For this reason, we decided to test the effect that this retinoid and its combination with CIN have on tumour growth in vivo. Orally treatment of a LA-N-1 xenograft model with 13-*cis*-RA alone or in combination with CIN did not alter survival curves of LA-N-1 xenograft models (p=1, Figure 27A). As expected, VEH treated tumours had an exponential growth curve. In the group of tumours treated with CIN there were two tumours that were treated for 13 week and one of them never reached the end volume of 1500mm$^3$. In addition, growth curves of tumours that received 13-*cis*-RA alone could be grouped in slow- and fast-growing tumours. Most tumours treated with an oral combination of CIN and 13-*cis*-RA had a growth curve similar to the tumours treated with VEH. Though, two tumours showed a slower growth, one of them was in treatment for more than 10 weeks to reach end volume of 1500mm$^3$ (Figure 27B, left panel). Analysis of tumour histology didn't reveal major differences between treatment groups. However, tumours that received CIN alone or in combination with 13-*cis*-RA had more necrotic areas than tumours treated with VEH (Figure 27B, right panel).
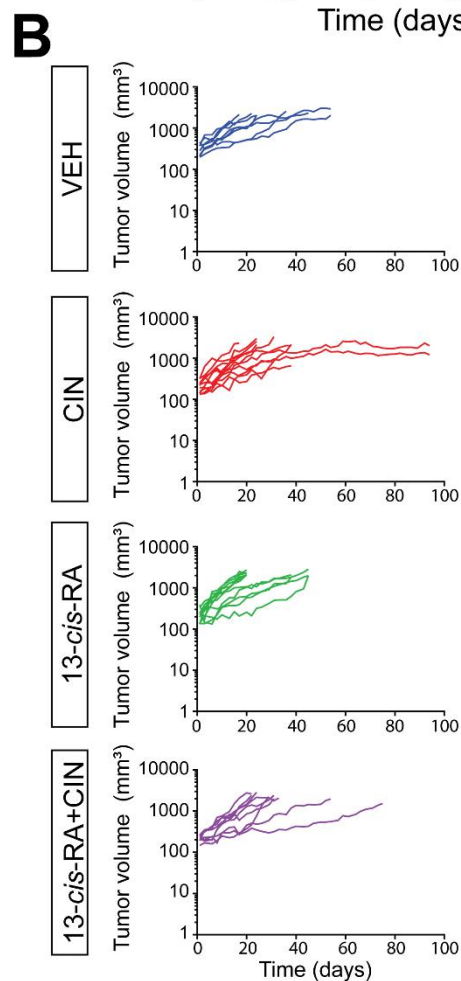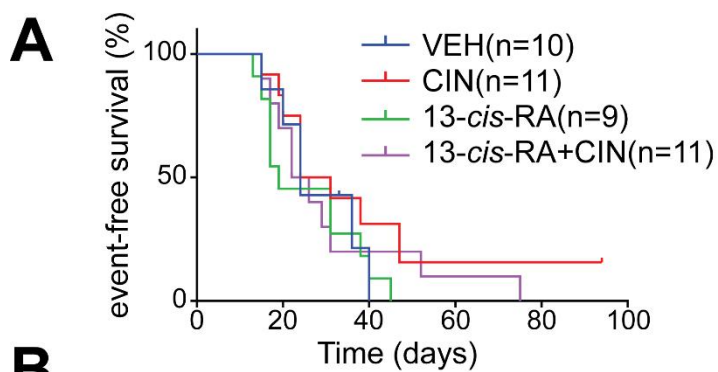
**Figure 27 – Effect of 13-cis-RA and its combination with CIN in NB tumour growth.** LA-N-1 cells were injected subcutaneously into each flank of four to six-old athymic nude-Foxn 1 *nu/nu* mice. When tumours reached a volume between 150-200mm$^3$ mice they were randomly assigned to each treatment group and receive daily an oral dose of 30mg/kg 13-*cis*-RA, 10mg/kg CIN alone or in combination or their vehicle (VEH). Animals were treated until tumours reached 2000mm$^3$ or after 10 weeks of treatment. **(A)** Kaplan-Meier event-free survival curves of mice treated with ATRA, CIN alone or in combination and with their vehicle. Event was considered reaching 1500mm$^3$, and log-rank test was used to compare the survival curves between groups. **(B)** Representation of individual tumour growth in each treatment group.

# 9        New calcimimetic for neuroblastoma treatment

Using CIN to target CaSR in NB was shown to be an interesting strategy for the treatment of this paediatric cancer. Our group demonstrated that this calcimimetic induced ER stress mediated apoptosis of NB cell lines. More importantly, CIN was capable of inhibiting *in vivo* tumour growth. However, these encouraging results came with an associated side effect, since CIN reduced plasma $Ca^{2+}$ levels, which it is complicated to manage in very debilitated patients (*Rodríguez-Hernández* et al – *2016*). Biased signalling is the ability of different GPCR ligands stabilize alternative receptor structures eliciting different intracellular responses (*Leach* et al – *2016*). Considering the biased signalling properties of CaSR, we hypothesized that other calcimimetics might have tumour suppressive properties similar to CIN without affecting plasma $Ca^{2+}$ levels.

## 9.1   AC-265347 maintains plasma calcium levels and inhibits NB tumour growth

In the same year two papers were published where authors demonstrated that two different calcimimetics, calcimimetic B and AC-265347, reduced PHT levels without causing hypocalcaemia in healthy rats (*Ma* et al – *2011*, *Henley III* et al – *2011*). Although both drugs could be interesting alternatives to CIN for the treatment of NB, we selected AC-265347 due to its commercial availability. First, we evaluated the effect that AC-265347 and CIN had in the plasma concentration levels of our mouse model. We treated healthy immunocompromised nude mice with an oral dose of 10 mg/kg/day of CIN or AC-265347 and evaluated ionised plasma $Ca^{2+}$ levels once per week, for 4 weeks. After one week of treatment with both calcimimetics, CIN significantly reduced plasma $Ca^{2+}$ levels relative to the vehicle treated mice (p=0.0142). However, no effect was observed when animals were treated with AC-265347. After two weeks of treatment, plasma $Ca^{2+}$ levels of CIN were not only significantly lower than VEH treated animals but also relative to AC-265347 (CIN vs VEH p=0.0455, CIN vs AC-265347 p=0.0591). In the consecutive weeks there was a tendency of CIN to reduce ionised plasma $Ca^{2+}$ (week 3, p=0.1405; week 4, p=0.0509), while AC-265347 showed no effect in the concentration of $Ca^{2+}$ in the blood after prolonged exposure to the drug (Figure 28A).
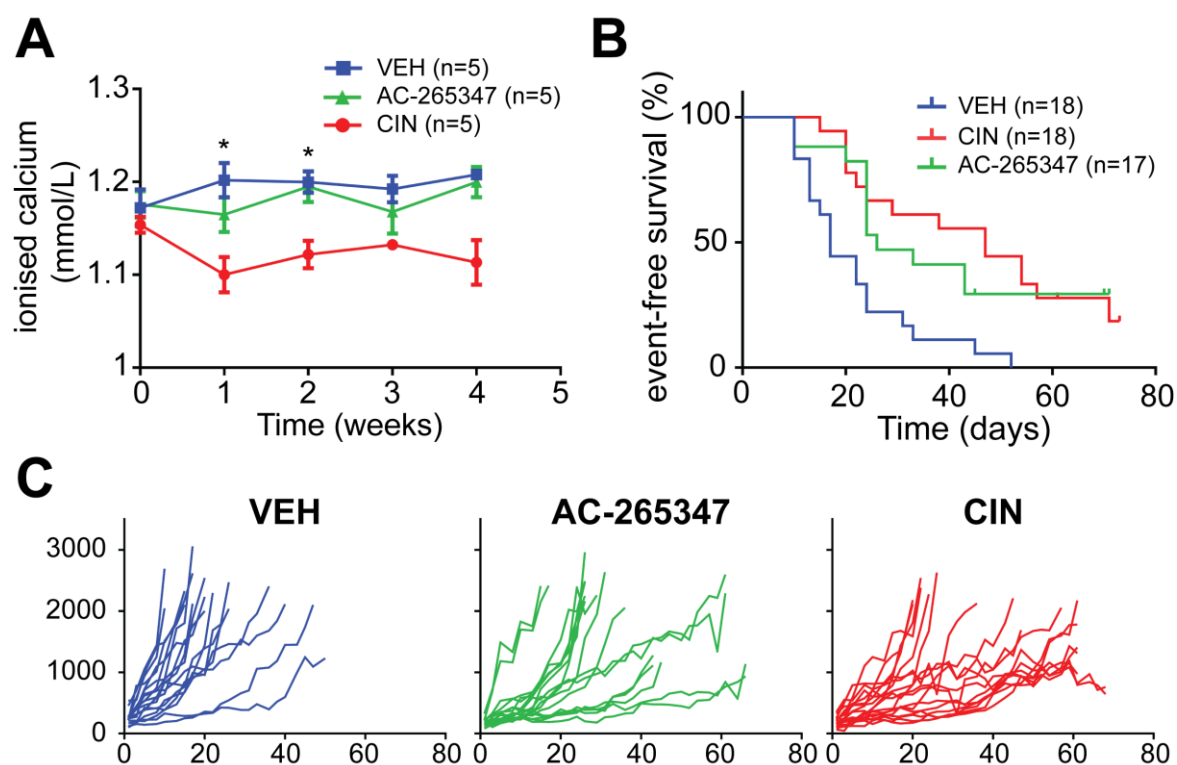
**Figure 28 – AC-265347 maintains plasma Ca²⁺ levels and inhibits NB tumour growth.**
**A –** Healthy athymic Nude-Foxn1 *nu/nu* mice, were treated with 10mg/kg of AC-265347, CIN or their vehicle. Blood was collected weekly from tail-vein and ionised plasma $Ca^{2+}$ levels were measured using an EPOC™ reader. Each point represents mean ± SEM of each group. Statistical significance at each week was calculated between treatment groups using One-way ANOVA with Dunn's correction. *p<0.005 **B –** Kaplan-Meier EFS curve of athymic Nude-Foxn1 *nu/nu* mice bearing LA-N-1 xenografts, treated with 10mg/kg of AC-265347, CIN or their vehicle. Log-rank statistics with a Bonferroni correction test was used to compare statistical significance between treatment groups (p=0.0186, VEH vs AC-265347; p=0.0003, VEH vs CIN; p=1, CIN vs Ac-265347). **C –** Representation of individual tumour growth in each treatment group.

Next, we aimed to explore the anti-tumorigenic properties of AC-265347 using a xenograft NB model. In order to compare CIN and AC-265347, we treated immunocompromised nude mice bearing a LA-N-1 xenograft with 10 mg/Kg/day of AC-265347, CIN or their VEH. Both calcimimetics significantly inhibited NB tumour growth (CIN vs VEH p=0.0003; AC-265347 vs VEH p=0.0186). In addition, no significant different EFS curves were obtained when tumours were treated with either CIN or AC-265347 (Figure 28B). As expected, analysis of individual tumours showed that VEH treated tumours presented a fast growth, with the exception of two tumours that only reached end-volume after day 40 of treatment. Xenograft tumours treated with AC-265347 showed very variable growth curves. However, it was possible to group them in two groups, fast and slow growth tumours and some tumours were

collected before reaching end for ethical reasons. Growth curves of tumours treated with CIN were more homogenous, however, some had a fast growth curve (Figure 28C).
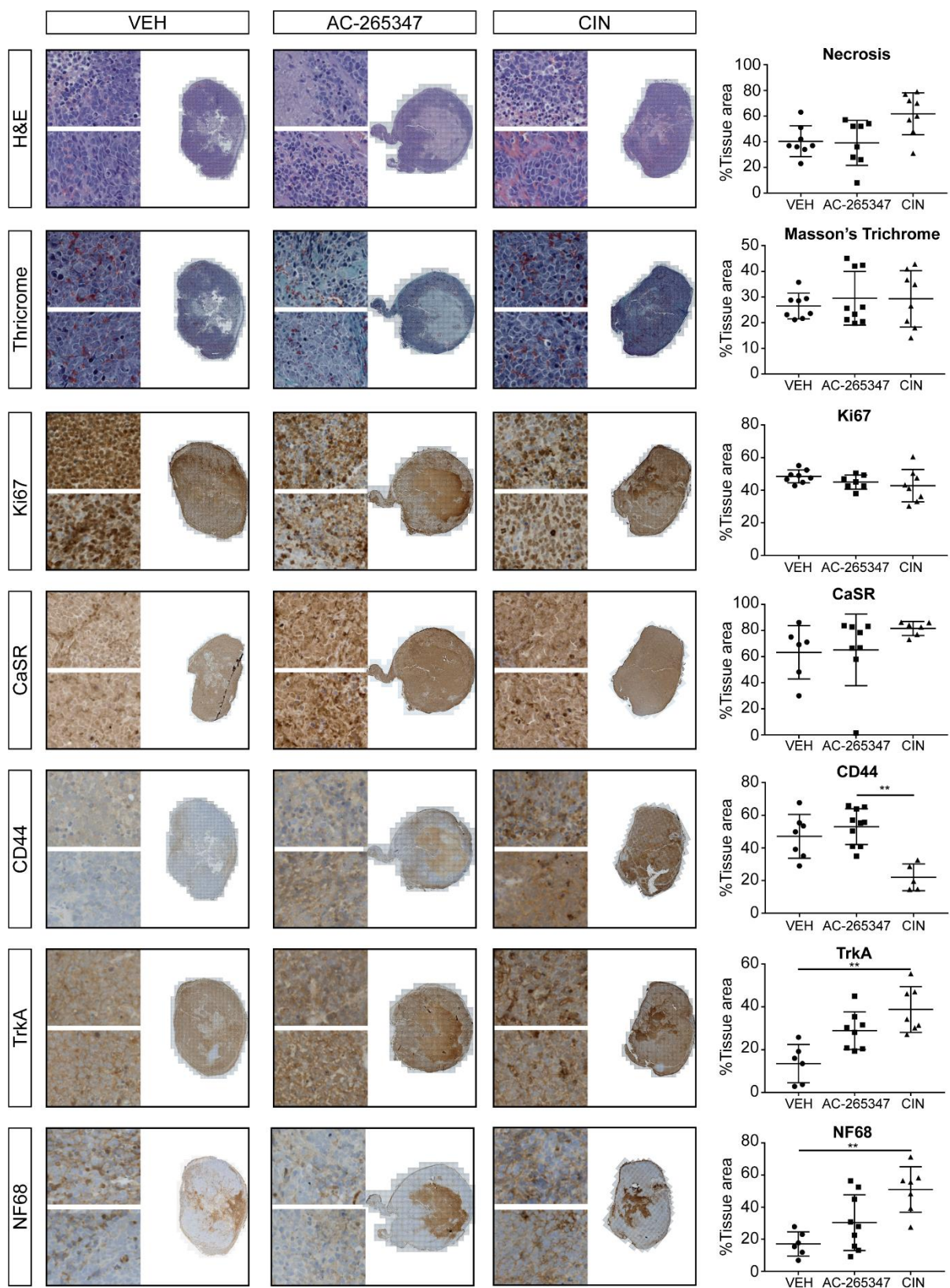
**Figure 29 – *In vivo* treatment with calcimimetic treatment increases levels of NB differentiation markers.** Formalin fixed, paraffin embedded sections of the six LA-N-1 xenografts exposed the longest to each treatment were stained with H&E, Masson's Trichrome, anti-Ki67, anti-CaSR, anti-CD44, anti-TrkA and anti-NF68. Necrosis quantification was performed under blinded conditions. Images were acquired with TissueFAXS at x20 magnification and percentage of positive area per tumour was quantified using STRATA Quest. Green and brown signal were considered as positive signal for Masson's Trichrome staining and IHC, respectively. Each symbol represents an individual tumour and lines represent mean ± SD. Significance was calculated using One-way ANOVA using a Dunn's correction test. **p<0.01. Scale represents 40µM.

## 9.2 Expression of NB differentiation markers is increased by CIN treatment

In order to study histological features of the treated tumours, we selected those exposed for the longest time to the two calcimimetics. Quantification of necrosis percentage present in each tumour, showed no differences between VEH and AC-265347 treated mice. On the contrary, CIN induced 20% more necrosis (p=0.0643, VEH vs CIN). Using TissueFAXS, an automated image acquisition system, and objective quantification by STRATA Quest software, we quantify Masson's trichrome staining, corresponding to the fibrotic area. Although some tumours showed close to 50% of fibrotic area, there was great variability in tumours from the same treatment group, preventing any statistical significance. Moreover, tumours generated by LA-N-1 cells presented very compact cells which does not allow for a correct cell segmentation by STRATA Quest software. Thus, we decided to quantify the expression of the different prognosis marker by detecting the stained area present in each tumour tissue. To avoid non-specific staining, we removed the higher expression areas, since it is known that antibodies bind non-specifically to necrotic areas (Figure 29). Quantification of Ki67 expression, a cell proliferation marker, showed its levels were similar in tumours treated with calcimimetics or VEH.

Next, we evaluated the effect that these calcimimetics had on CaSR protein expression. Tumours treated with CIN showed an upregulation of CaSR expression, albeit this was not statistically significant (p=0.1711). However, no effect was observed when tumours were treated with AC-265347. In addition, we evaluated the expression of three different NB prognosis markers, CD44, TrkA and NF68. Each of them was affected differently by the two calcimimetics used. CD44 expression was 2.1-fold reduced when LA-N-1 xenografts were treated with CIN (CIN vs VEH

p=0.0506) while no effect was induced by AC-265347. Expression of TrkA and NF68 was significantly upregulated by CIN (approximately 2- and 3-fold, respectively) (Figure 29).

## 9.3 AC-265347 and CIN induce a different gene expression pattern in a NB xenograft model

Our group previously reported that CIN altered gene expression pattern of LA-N-1 xenograft models (*Rodríguez-Hernández* et al – *2016*). Thus, to compare the effect induced by AC-265347, we evaluated the mRNA expression levels of eight of the most responsive LA-N-1 xenografts. First, we evaluated the effect of these calcimetics on *CaSR* mRNA levels. AC-265347 and CIN induced a higher than 2-fold increase in the *CaSR* mRNA expression, however this up-regulation was not significantly different. In addition, only CIN induced a 2.26-fold upregulation of *RYR* mRNA, gene involved in calcium signalling quantification (CIN vs VEH p=0.1426) (Figure 30).

Expression of genes associated with NB differentiation was highly modified by exposure to calcimimetics. Relative to the VEH treated xenografts, CIN significantly increased the mRNA expression of *NEFL*, *TrkA*, *TUBB3* and CHD5 (2.31, 3.57, 5.24, 11-91-fold increase, respectively). Both calcimimetics induced a 1.5-fold reduction of the mRNA expression of *NGFR* (p=0.0332, VEH vs AC-265347; p=0.0559, VEH vs CIN). *In vivo* exposure to these calcimimetics did not modify their expression of *MYCN* or *ID2*, two genes associated with NB aggressiveness. However, expression of *ID2* was significantly different between tumours treated with AC-265347 and CIN (p=0.0194, CIN vs AC-265347). Moreover, AC-265347 reduced the expression of genes associated with ER stress and apoptosis, albeit only *NOXA* expression is statistically reduced (2.5-fold, p=0.0119, VEH vs AC-265347) while *PUMA* (p=0.0589, VEH vs AC-265347) and *ATF4* (p=0.0778, VEH vs AC-265347) were tendentially lower expressed. On the contrary, exposure to CIN increased the expression of *CHOP* (1.58-fold, p=0.1491, VEH vs CIN) and *BID* (2.15-fold, p=0.0216, VEH vs CIN), and decreased the expression *PUMA* (1.4-fold, p=0.024, VEH vs CIN) and *NOXA* (2.3-fold, p=0.1061, VEH vs CIN). Expression of *CHOP* and *BID* was significantly different between tumours treated with CIN or AC-265347 (CHOP p=0.0008, CIN vs AC-265347; BID p=0.0016, CIN vs AC-265347) (Figure 30).

| Category | Gene | AC-265347 (n=8) | CIN (n=8) |
|---|---|---|---|
| Ca²⁺ signalling | CaSR | 2.51 | 2.12 |
| | PRKCA | 0.85 | 1.15 |
| | RYR | 1.15 | 2.26 |
| | ADCY8 | 1.08 | 1.28 |
| Differentiation | NEFL | 1.47 | 2.31 * |
| | TrkA | 1.76 | 3.67 * |
| | TrkC | 1.25 | 1.81 |
| | NGFR | 0.63 * | 0.61 |
| | S100β | 1.31 | 1.51 |
| | TUBB3 | 1.41 | 5.24 ** |
| | CD44 | 0.98 | 1.20 |
| | CHD5 | 1.10 | 11.91* |
| Proliferation | MYCN | 1.09 | 0.93 |
| | ID2 | 0.85 | 1.46 |
| ER stress | GRP78 | 0.89 | 0.97 |
| | CHOP | 0.78 | 1.58 |
| | Trib3 | 0.85 | 0.95 |
| | ATF4 | 0.76 | 1.07 |
| | PUMA | 0.50 | 0.70 * |
| | NOXA | 0.40 * | 0.43 |
| | BID | 0.91 | 2.15 * |
| Epithelial mesenchymal transition (EMT) | Col1A1 | 2.18 | 1.64 |
| | Col3 | 0.64 | 0.95 |
| | TGFβ | 1.56 * | 1.44 |
| | TGFβ2 | 1.16 | 1.58 |
| | TGFβ3 | 1.42 | 1.18 |
| | TGFβR | 0.88 | 0.69 |
| | EGF | 1.28 | 1.75 |
| | EGFR | 1.09 | 1.25 |
| | VEGF | 1.18 | 0.99 |
| | CTGF | 0.47 | 0.28 |
| | VIM | 0.93 | 1.28 |
| | MMP2 | 1.05 | 0.51 * |
| Cancer testis agents (CTAs) | CTCFL | 1.90 | 2.47 |
| | SSX4/4B | 3.05 | 10.64 |
| | GAGE12J | 3.56 | 9.90 *** |
| | MAGEA2 | 1.01 | 0.98 |
| | MAGEA3 | 1.16 | 2.86 |
| | MAGEA6 | 1.40 | 2.48 * |
| | NY-ESO-1 | 1.68 | 5.24 |

Scale: 1 5 10

**Figure 30 – AC-265347 and CIN induce differential gene expression in *in vivo* LA-N-1 xenograft mouse model.** RNA was extracted from the last eight tumours exposed to the longest treatment period. mRNA levels of the indicated genes were quantified by RT-qPCR and normalised to LA-N-1 expression. Indicated values represent fold difference between mean expression of each group, relative to mean expression of vehicle treated tumours. Statistical significance was calculated between means of each group using one-way ANOVA with a Dunn's correction. *p<0.05, **p<0.01, ***p<0.001, relative to vehicle treated tumours.

Next, we evaluated the effect that calcimimetics had on the mRNA expression of several genes associated with epithelial mesenchymal transition (EMT), an important process for metastatic and invasion capacities of cancer cells (Dongre et Weinberg-2018). Amongst genes related with EMT, only *MMP2* and *TGFβ* were significantly modified in tumours treated with either calcimimetic. We

observed that AC-265347 significantly increased the expression of *TGFβ* (1.56, p=0.034, VEH vs AC-265347), while CIN strongly reduced the mRNA levels of *MMP2* (1.96-fold, p=0.0134, VEH vs CIN). Of note, *Col1A1* mRNA expression was upregulated more than 2-fold in tumours treated with AC-265347, albeit this was not statistically significant (p=0.243, VEH vs AC-265347) (Figure 30).

We previously demonstrated that CIN increases the expression of cancer testis antigens (CTAs) in a mouse model of NB (*Rodríguez-Hernández* et al – *2016*). In the present work we validated this finding, since we observed that tumours treated with CIN had a significantly higher expression of *CTLCF* (2.47-fold, p=0.0216), *GAGE12J* (9.9-fold, p=0.0012) and *MAGEA6* (2.48-fold, p=0.0194), relative to VEH treated tumours. On the contrary, treatment with AC-265347 had a mild effect on the expression of CTAs, because although there was a 3-fold upregulation of *SSX4/4B* and *GAGE12J*, this was not statistically significant (Figure 30).

## 9.4 NB patient derived xenograft (PDX) models express CaSR and respond differently to calcimimetics

To evaluate the effect of these two calcimimetics in a more physiological tumour model, we used NB PDX models generated by Dr. Angel M. Carcaboso's research group of the *Institut de Recerca Sant Joan de Déu* (HSJD) (Monterrubio et al-2015). Assessment of CaSR protein expression showed that all PDX expressed CaSR dimer (band at approximately 240kDa) and some of those presented a low intensity band corresponding to the CaSR monomer (at approximately 140kDa). Highest levels of CaSR were detected on HSJD-NB001, while HSJD-NB007 showed the lowest levels of this protein. Thus, we decided to use HSJD-NB001, HSJD-NB004 and HSJD-NB012 for our studies since they have both high CaSR protein levels and high engraftment rate, being success rate higher than 50% (Figure 31A).

First, we analysed the EFS curves of the three PDX models treated with CIN and AC-265347. Growth of HSJD-NB001 xenografts was significantly inhibited by the two calcimimetics (CIN vs VEH p=0.0081; AC-265347 vs VEH p=0.0105), while VEH treated tumours took on average 11 days to reach end-volume, AC-1265347
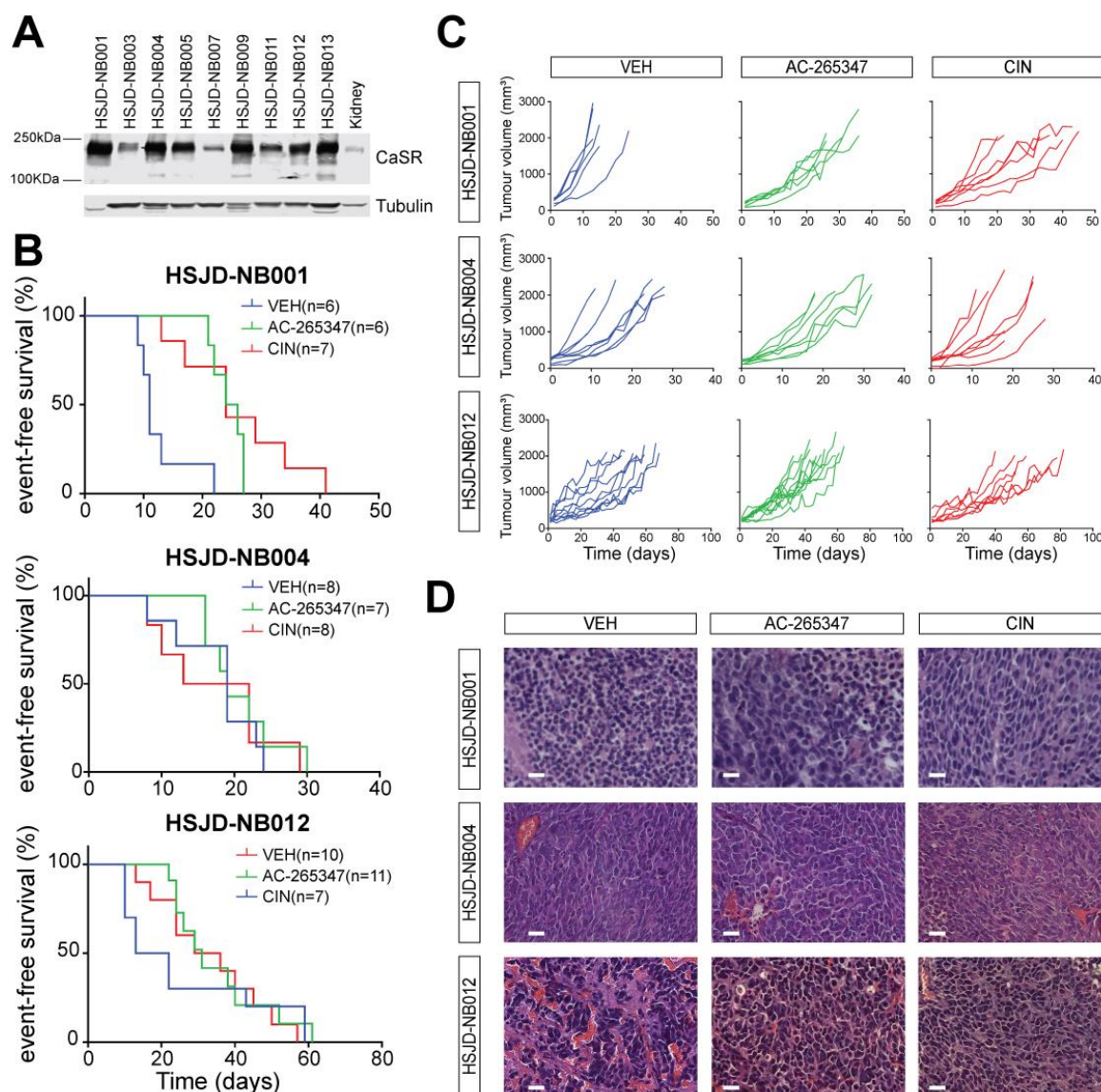


**Figure 31 – Effect of calcimimetics in NB PDX models. A –** CaSR protein levels of NB PDX models. **B –** EFS curve of athymic Nude-Foxn1 *nu/nu* mice bearing the indicated PDX, treated with 10mg/kg of AC-265347, CIN or their vehicle. Log-rank statistics with a Bonferroni correction test was used to compare statistical significances between treatment groups HSJD-NB001 (p=0.0105, VEH vs AC-265347; p=0.0081, VEH vs CIN; p=1, CIN vs Ac-265347), HSJD-NB004 (p=1 for all comparisons), HSJD-NB012 (p=0.912, VEH vs AC-265347; p=1, VEH vs CIN and CIN vs AC-265347). **C –** Representation of individual tumour growth of the three PDX models treated with CIN, AC-265347 or VEH. **D –** H&E stained sections of PDX models treated with each calcimimetic or VEH. White bar represents 40µm (x20 magnification).

and CIN took 25 and 24 days, respectively  (Figure 31B). Analysis of single tumour growth curves showed a more gradual growth in HSJD-NB001 xenografts treated with either CIN or AC-265347. HSJD-NB004 tumours reached the end-volume after 30 days of treatment irrespective of calcimimetics or not, whereas HSJD-NB012 tumours presented a highly variably growth curves, (Figure 31C). Analysis of H&E staining of PDX tumours treated with calcimimetics showed different histological features between tumour models, but none between treatments. The exception was the HSJD-NB001 PDX model, where tumours treated with CIN and AC-26534 had cells with a larger cytoplasm and larger nuclei, than VEH treated tumours suggesting a more differentiated phenotype (Figure 31D).

### 9.5   CIN promotes a more differentiated phenotype than AC-265347 in HSJD-NB001

In order to elucidate the mechanisms underlying the tumour suppressive effect of both calcimimetics on the HSJD-NB001 PDX model, we used the same approach described in section 9.2 to evaluate fibrosis and protein expression of cell proliferation and differentiation markers. Tumours treated with AC-265347 showed a 1.5-fold lower percentage of fibrotic area (AC-265347 vs VEH p=0.3353) than VEH or CIN treated tumours, although there was high variability between treated tumours. In addition, the cell proliferation marker Ki67 showed no alteration when tumours were treated with either calcimimetic. HSJD-NB001 tumours treated with AC-265347 showed a slightly higher CaSR positive tissue area than VEH or CIN treated tumours (1.28-fold, AC-265347 vs VEH p=0.5468), however we observed high variability (Figure 32).

Evaluation of different NB differentiation markers showed no changes in

**Figure 32 – Histological evaluation of HSJD-NB001 tumours treated with CIN and AC-265347.**  Formalin fixed, paraffin embedded sections of HSJD-NB001 tumours exposed to each treatment were stained with Haemotoxilin-eosin (H&E), Masson's Trichrome, anti-Ki67, anti-CaSR, anti-CD44, anti-TrkA and anti-NF68. Images were acquired with TissueFAXS at x20 magnification and percentage of positive area per tumour was quantified using STRATA Quest. For Masson's Trichrome staining green signal was considered as positive signal, while for IHC staining brown staining was considered as a positive.  Each symbol represents an individual tumour and lines represent mean ± SD. Significance was calculated using One-way ANOVA with a Dunn's correction test. *p<0.05. Scale represents 40µM.

CD44 protein expression levels induced by any calcimimetic. However, treatment of HSJD-NB001 with both calcimimetics resulted in a upregulation of TrkA and NF68 protein levels, albeit statistical significance was only found in tumours treated with
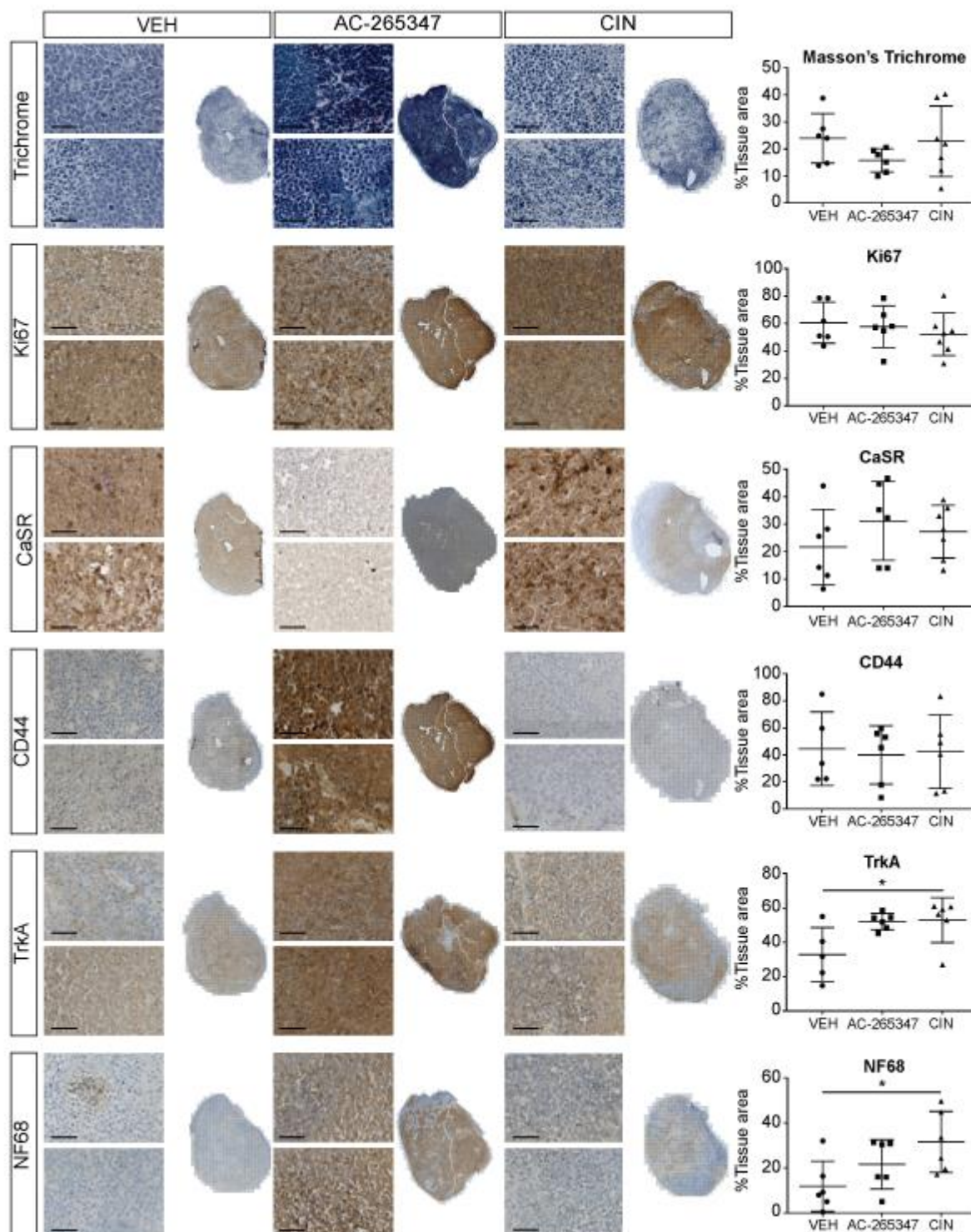


CIN (CIN vs VEH, TrkA 1.5-fold p=0.0480 and NF68 2.6-fold p=0.0449; AC-265347 vs VEH, TrkA 1.5-fold p=0.4146 and NF68 1.82-fold p=0.9912) (Figure 32).

### 9.6 AC-265347 and CIN induce different pattern of gene expression in HSJD-NB001

In order to compare if both calcimimetics induced similar effects on HSJD-NB001 than those observed in LA-N-1 xenografts, we analysed the mRNA expression of genes reported in section 6.3. In agreement with LA-N-1 xenografts, *CaSR* mRNA levels showed an upregulation induced by either AC-265347 or CIN (17.33 and 15.46-fold increase, respectively), however this was not statistically significant (AC-265347 vs VEH p= 0.0995; CIN vs VEH p=0.1198). In contrast to LA-N-1 xenografts, expression of genes involved in calcium signalling were not modified by treatment with both calcimimetics. Similar to LA-N-1 xenografts, CIN induced a 1.98-fold upregulation of both *NEFL* (p=0.3143) and *S100β* (p=0.5831) in the HSJD-NB001 model. Surprisingly, treatment with either AC-265347 or CIN induced a 3-fold increase of *CD44* mRNA levels (p=0.4887 and p=0.157, respectively). More importantly, AC-265347 significantly reduced mRNA levels of *MYCN* (1.47-fold, p=0.0283). None of the genes associated with ER stress were significantly modified by treatment with either calcimimetic. However, AC-265347 increased the expression of *PUMA* more than 2-fold (VEH vs AC-265347p=0.3505, VEH vs AC-265347) (Figure 33).

Treating HSJD-NB001 tumours with AC-265347 reduced more than 2-fold the expression of genes related with EMT such as *ColA1A* (p=0.1547, AC-265347 vs VEH), *TGFβ* (p=0.1547, AC-265347 vs VEH) and *TGFβ2* (p=0.0175, AC-265347 vs VEH). In contrast with LA-N-1 xenografts, tumours treated with this calcimimetic increased more than 3-fold mRNA expression of *Col3* (p=0.99, AC-265347 vs VEH). Accordingly, treatment of this tumour model with CIN lead to a nearly 2-fold reduction of the mRNA levels of *ColA1A*, *MMP2* and *VIM* (p=0.4329, p=0.1197 and p=1, CIN vs VEH, respectively). Furthermore, we quantified the expression of CTAs, potential targets for immunotherapy, AC-265347 treatment increased mRNA levels of *SSX4/4B*, *MAGEA3*, *MAGEA6*, albeit not significantly. On the contrary, treatment with CIN induced a higher than 2-fold decrease of mRNA levels of *SSX4/4B* (p=0.3505), *GAGE12J* (p=1), *MAGEA2* (p=0.5831), *MAGEA3* (p=0.2231), *MAGEA6* (p=0.4272) and *NY-ESO-1* (p=0.7845) (all CIN vs VEH)(Figure 33).

| | | AC-265347 (n=6) | CIN (n=7) |
|---|---|---|---|
| **Ca²⁺ signalling** | CaSR | 17.33 | 15.46 |
| | PRKCA | 0.86 | 1.15 |
| | RYR | 1.16 | 1.75 |
| | ADCY8 | 1.34 | 0.69 |
| **Differentiation** | NEFL | 1.23 | 1.98 |
| | TrkA | 0.69 | 0.90 |
| | TrkC | 1.12 | 0.95 |
| | NGFR | 0.90 | 0.87 |
| | S100β | 1.23 | 1.98 |
| | TUBB3 | 0.94 | 0.82 |
| | CD44 | 2.93 | 3.14 |
| | CHD5 | 1.50 | 1.07 |
| **Proliferation** | MYCN | 0.68* | 0.90 |
| | ID2 | 1.15 | 0.54 |
| **ER stress** | GRP78 | 1.00 | 1.08 |
| | CHOP | 1.18 | 1.14 |
| | Trib3 | 1.27 | 1.40 |
| | ATF4 | 1.32 | 1.06 |
| | PUMA | 2.46 | 1.55 |
| | NOXA | 1.57 | 0.94 |
| | BID | 1.31 | 0.87 |
| **Epithelial mesenchymal transition (EMT)** | Col1A1 | 0.39 | 0.51 |
| | Col3 | 3.90 | 1.77 |
| | TGFβ | 0.44 | 0.92 |
| | TGFβ2 | 0.45* | 0.64 |
| | TGFβ3 | 0.80 | 1.03 |
| | TGFβR | 1.21 | 1.51 |
| | EGF | 0.78 | 0.94 |
| | EGFR | 0.86 | 0.63 |
| | VEGF | 0.69 | 0.84 |
| | CTGF | 0.58 | 1.36 |
| | VIM | 0.68 | 0.55 |
| | MMP2 | 1.05 | 0.51 |
| **Cancer testis agents (CTAs)** | CTCFL | 1.22 | 1.00 |
| | SSX4/4B | 2.79 | 0.69 |
| | GAGE12J | 0.32 | 0.13 |
| | MAGEA2 | 0.92 | 0.45 |
| | MAGEA3 | 3.50 | 0.49 |
| | MAGEA6 | 2.36 | 0.43 |
| | NY-ESO-1 | 1.32 | 0.43 |

**Figure 33 – Gene expression of HSJD-NB001 tumours treated with calcimimetics.**
RNA was extracted from all the tumours treated with CIN, AC-265347 or VEH. mRNA levels of the indicated genes were quantified by RT-qPCR and normalised to the mean of those detected in VEH control tumours. Indicated values represent fold difference between mean expression of each treatment group, relative to mean expression of vehicle treated tumours. Statistical significance was calculated between means of each group using one-way ANOVA with a Dunn's correction. *$p<0.05$, **$p<0.01$, ***$p<0.001$, relative to vehicle treated tumours.

## 9.7 Calcimimetics maintained NB cell viability

Our group previously reported that CIN did not exert a cytotoxic effect on NB cell lines (*Rodríguez-Hernández* et al – *2016*). In order to elucidate the mechanism of action of AC-265347 *in vitro,* we first calculated the half maximal inhibitory

concentration ($IC_{50}$) values of AC-265347 for six NB cells lines, two of which expressed low endogenous levels of CaSR (LA-N-1 and IMR5). The more sensitive cell line was SH-SY5Y with an $IC_{50}$ value of 29.66µM. However, this concentration was too high to be considered as a cytotoxic. The two NB cell lines that showed a low endogenous expression of CaSR also had an elevated $IC_{50}$, making us reason that similar to CIN, AC-265347 does not exert a cytotoxic effect on NB cell lines (Figure 34A, Table 11).

| Cell line | Phenotype | MYCN status | TP53 status | AC-265347 $IC_{50}$ |
|-----------|-----------|-------------|-------------|---------------------|
| LA-N-1 | N-type | amplified | Mutated | 43.53µM |
| SH-SY5Y | N-type | non-amplified | wild-type | 29.66µM |
| IMR5 | N-type | amplified | wild-type | 50.5µM |
| SK-N-LP | I-type | amplified | wild-type | 39.94µM |
| SK-N-JD | I-type | Amplified | wild-type | 45.23µM |

**Table 11 –** AC-265347 $IC_{50}$ values from NB cell lines

To further validate that AC-265347 did not induce cytotoxicity in NB cells, we treated LA-N-1 and IMR5 stably transfected with CaSR or the mock plasmid (GFP) with 1µM of CIN, AC-265347 or their vehicle (DMSO). LA-N-1-GFP-CaSR showed a significantly lower proliferation rate than cells transfected with the mock plasmid (GFP) (day3 p=0.0353, day4 p=0.0268, LAN-GFP DMSO vs LAN-GFP-CaSR DMSO). However, treating transfected cells with either calcimimetic did not significantly reduced cell viability relative to DMSO treated cells (Figure 34B).

In IMR5 cells stably transfected with CaSR, treatment with either CIN or AC-265347 resulted in a significantly higher viability relative to GFP cells (day2 p=0.0113, day3 p=0.0137, LAN-GFP AC-265347 vs LAN-GFP-CaSR AC-265347). However, a longer exposure to calcimimetics did not affect cell viability. Differences in viability were mainly due to the overexpression of CaSR and not to the activation of this GPCR with calcimimetics (Figure 34C).
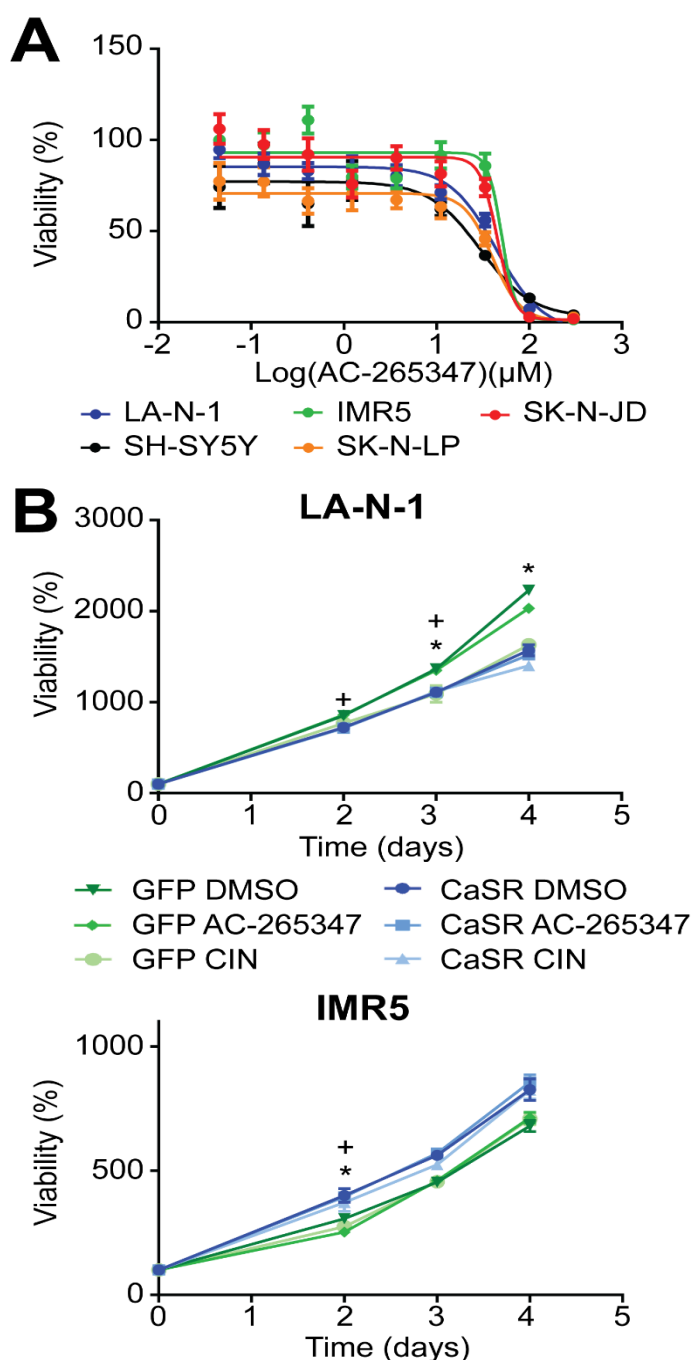
**Figure 34 – NB cell viability is reduced by CaSR overexpression (A)** Cytotoxic activity of AC-265347 of five neuroblastoma cell lines was done by treating the indicated cell lines with a consecutive lower concentration of AC-265347 (starting at 100µM). Each point represents mean ± SEM of six technical replicates and IC50 was calculated using GraphPad and is indicated in table 1. **(B)** LA-N-1 and IMR5 cells were stably transfected with pGFP-CaSR or pGFP and treated with 1µM of AC-265347, CIN or their vehicle (DMSO) for 5 days. Cell viability was measured after 2, 3, 4 and 5 days, and percentage of viability was normalized to the number of viable cells on day 0. Each point represents mean ± SEM of six technical replicates. Statistical significance between groups in each day was calculated using One-way ANOVA with Dunn's correction test. *p<0.05 GFP DMSO vs CaSR DMSO; + p<0.05 GFP AC_265347 vs CaSR AC265347.

## 9.8   NB cell lines respond differently to prolonged exposure to CIN and AC-265347

Inhibition of NB tumour growth was observed only over prolonged exposure to calcimimetics. To elucidate the underlying molecular mechanism, we treated LA-N-1 and IMR5 cells with 1µM AC-265347, CIN or DMSO, for 14 days. LA-N-1 cells treated with CIN showed longer dendrites than DMSO control cells, while no morphological differences were observed when treated with AC-265347. On the

contrary, IMR5 cells treated with AC-265347 showed more complexed dendrites and flatter colonies than DMSO or CIN treated cells (Figure 35A).

We evaluated the mRNA expression of NB differentiation and stem-like associated genes. In LA-N-1 cells, *CaSR* expression was not affected by calcimimetics. Moreover, AC-265347 induced a mild effect in the expression of NB differentiation markers (*TUBB3*, *NGFR* and *TrkA*). In addition, both calcimimetics induced approximately 20% reduction in the levels of *CD44* mRNA, while LA-N-1 cells had a 25% reduction in *S100β* mRNA levels, as compared to DMSO control cells. Evaluation of genes associated with a stem-like phenotype showed that CIN downregulated approximately 25% of *Nanog*, *HES1* and *SOX2* mRNA expression. The effect of AC-265347 on the expression of *Nanog*, *HES1*, *Oct4* and *Sox2* was less than 20% reduction (Figure 35B, left panel).

IMR5 cells were more responsive than LA-N-1 to calcimimetics, both CIN and AC-265347 increased more than 2.5-fold the levels of *CaSR* mRNA. AC-265347 upregulated more than 1.5-fold the expression of *NEFL*, *S100β* and *NGFR*. CIN reduced the mRNA levels of *HES1* and *Sox2* more than 40%, however it did not modify the expression of NB differentiation genes. IMR5 cells treated with AC-265347 reduced more than 30% their expression of *Snail2*, gene involved in the epithelial-mesenchymal transition (Figure 35B, left panel).

Next, we evaluated the protein levels of NF68 (coded by the *NEFL* gene), TrkA and Sox2. The results obtained at protein level differ from results obtained analysing gene expression levels. LA-N-1 cells increased NF68 protein more than 2-fold after AC-265347 treatment, although treated cells maintained mRNA levels. This up-regulation was even higher after CIN treatment, approximately 5-fold. Additionally, CIN induced a 75% downregulation of TrkA in LA-N-1 cells while AC-265347 did not alter the levels of this protein. Expression of Sox2 was not modified by the treatment with either calcimimetic. IMR5 cells did not alter the levels of NF68, TrkA or Sox2 after AC-265347. On the contrary, IMR5 increased NF68 and TrkA more than 2-fold and reduced almost 30% Sox2 expression upon CIN treatment (Figure 35C).

Cell differentiation is accompanied by cell cycle arrest (*Liu* et al – *2019*). Thus, we explored if cell cycle arrest was the effect observed in NB cell lines upon calcimimetics treatment. Although quantification of the LA-N-1 cells in G1 phase showed a 10% increase relative to DMSO, this value was not significant. In addition,

a 50% of reduction in the G2 phase was observed when cells were treated with AC-
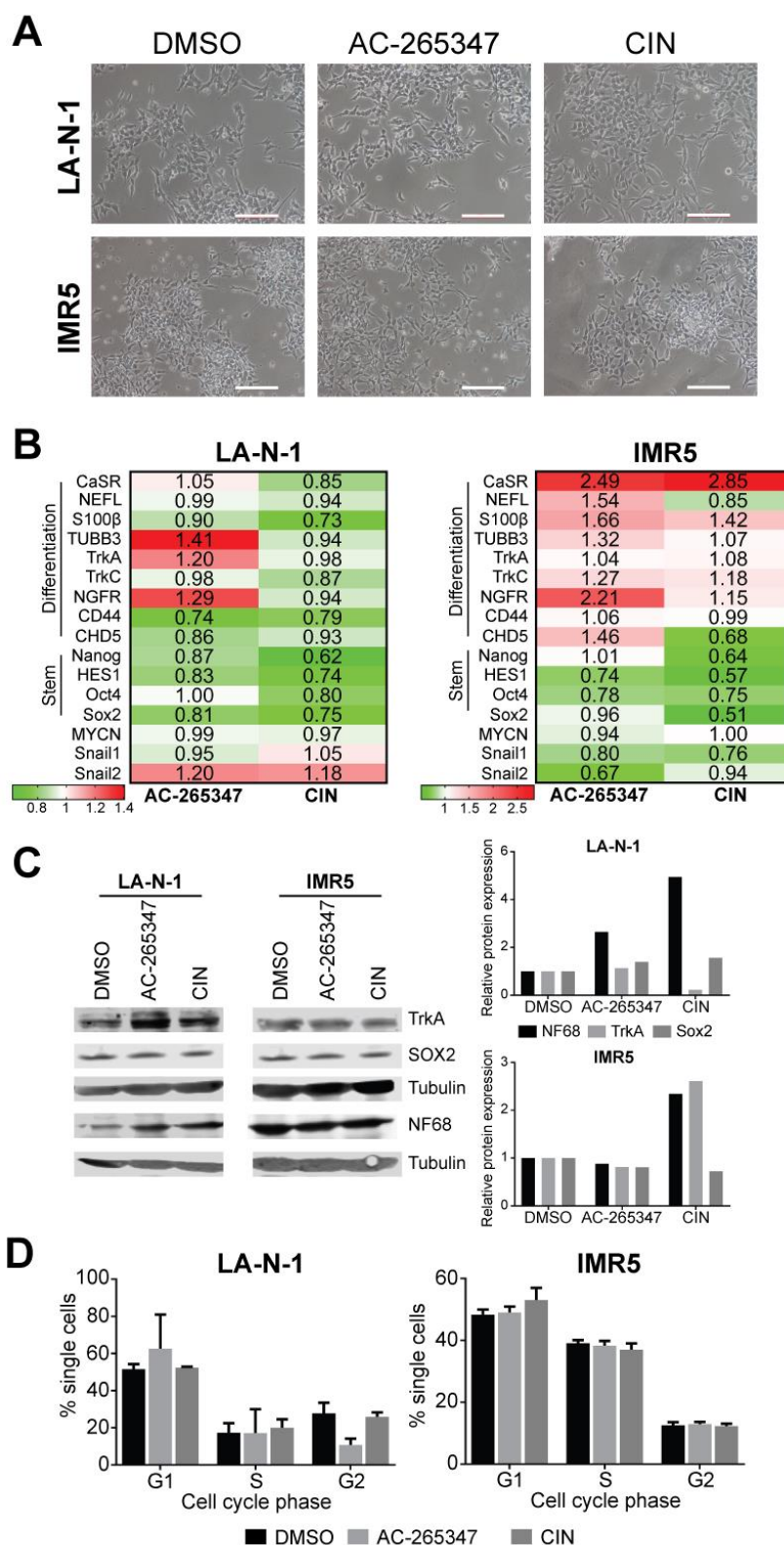


**Figure 35 – Prolonged exposure to calcimimetics alters NB differentiation markers. A –** Microphotography of LA-N-1 and IMR5 cells treated with 1µM of AC-265347, CIN or DMSO for 14-days **B –** Heat-map of relative mRNA expression levels of NB differentiation, stem cell and NB aggressive markers (MYCN, Snail1, Snail2) of LA-N-1 and IMR5 at experimental endpoint. Three independent experiments were conducted. Values represent fold difference between mean expression of treated cells relative to mean expression of DMSO control cells. Statistical significance was calculated using one-way ANOVA with Dunn's correction test. **C –** Immunoblot of TrkA, Sox2, NF68 and Tubulin of LA-N-1 and IMR5. Quantification of immunoblots was done using ImageJ and bars represent band intensity relative to anti-tubulin. **D –** Cell cycle analysis of NB cell lines treated for 14 days with calcimimetics was done using flow cytometry. Percentage of cells in each cell cycle stage was quantified using FlowJo and bars represent mean±SEM of four independent experiments.
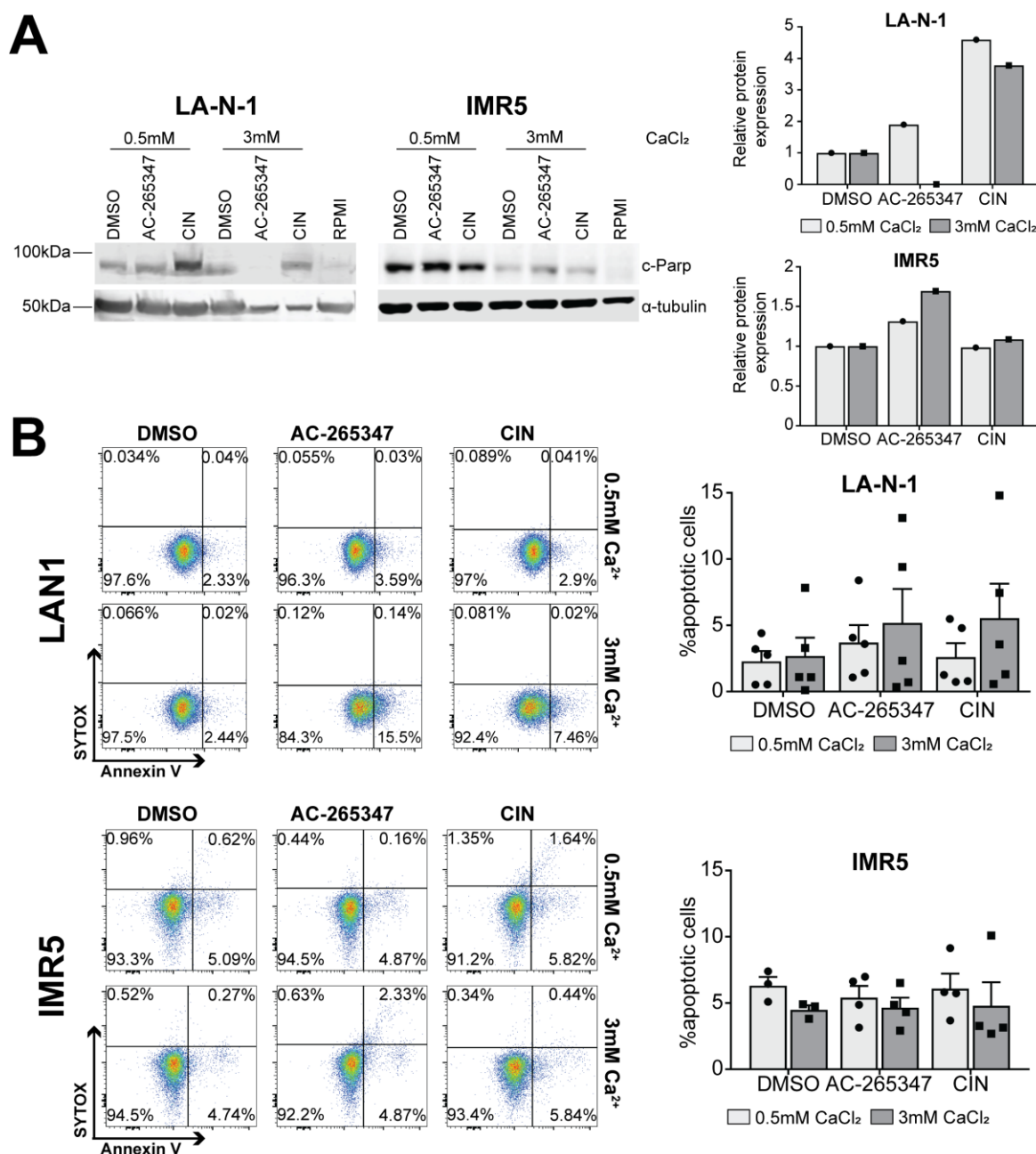
**Figure 36 – Acute exposure to AC-265347 affects differently NB cell lines. (A)** LA-N-1 and IMR5 were treated for 24-hours with 1µM of CIN, AC-265347 or their vehicle (DMSO) in the presence of 0.5 or 3mM of CaCl₂. After cell collection protein was extracted and immunoblot of cleaved PARP (c-PARP) was done. Band intensities were quantified relative to α-tubulin. **(B)** IMR5 and LA-N-1 cells were stained with SYTOX and Annexin V after a 16-hour treatment of 1µM of CIN, AC-265347 or their vehicle (DMSO) in the presence of 0.5 or 3mM of CaCl₂. Annexin V positive cells in each treatment were quantified by flow cytometry. Bar represent mean of four to five independent experiments and each point represents an individual experiment.

265347 (Figure 8D, left panel). Treating IMR5 cells with CIN or AC-265347 did not alter the proportions of cells in each cell cycle stage (Figure 35D, right panel).

### 9.9 Effects of acute exposure to CIN and AC-265347 in NB cell apoptosis

Our group previously described that exposure of NB cell lines to CIN in the presence of very low or high concentration of $Ca^{2+}$ induced ER mediated apoptosis (*Rodríguez-Hernández* et al – *2016*). Thus, we aimed at investigating if AC-265347 had a similar effect on NB cells lines. Assessment of cell death was done by quantification of cleaved PARP (c-PARP) protein in NB cells treated with $1\mu M$ of CIN, AC-265347 or DMSO. After 24hours of treatment, LA-N-1 cells treated with AC-265347 in the presence of 0.5mM of $Ca^{2+}$ showed a 2-fold increase of c-PARP protein levels relative to DMSO control cells. Accordingly, to published results, LA-N-1 cells treated with CIN increased the expression of c-PARP relative to DMSO both in the presence of 0.5mM and 3mM $Ca^{2+}$ (4- and 4.5-fold, respectively). Treating IMR5 with CIN maintained the levels of expression of c-PARP, when compared to DMSO control. A mild effect was observed (1.5-fold) when this NB cell line was treated with AC-265347 in the presence of high and low $Ca^{2+}$ concentrations (Figure 36A).

To further evaluate the divergent effect induced by the two calcimimetics, we quantified apoptosis using flow cytometry and the presence of extracellular annexin V after 16 hours of treatment. LA-N-1 cells grown in the presence of 0.5mM or 3mM $Ca^{2+}$ showed a low percentage of dead cells (around 2% for both conditions). When LA-N-1 were treated with AC-265347 in the presence of 0.5mM $Ca^{2+}$ there were 60% more apoptotic cells, as compared to DMSO control. However, this difference was not statistically significant due to high variability between assays. In addition, we observed that in the presence of high $Ca^{2+}$ concentrations CIN and AC-265347 induced cell death to the same extent, close to 90% (Figure 9B, top panel). IMR5 cells were found to be more sensitive to extreme concentration of $Ca^{2+}$, since there was about 5% of apoptotic cells in vehicle (DMSO) treated conditions. However, no effect was observed when treating IMR5 with either calcimimetic (Figure 36B, bottom panel).

# V. Discussion

# 10    Discussion

In the last 20 years, there has been an increase in the understanding of the biology of neuroblastoma allowing for more precise risk stratification, thus improving the outcome of many NB patients. In spite of this, there are about 50% of patients that do not survive this disease (*Whittle* et al – *2017, Matthay* et al – *2016*). Many patients respond to treatment at an initial stage and enter remission, however, some still suffer from relapsed or refractory disease and their survival rate is about 20% (*Zage* – *2018*). There is thus a need to develop new treatment strategies to prevent relapses and improve outcome. The most common approach is the use of differentiation agents, such as isotretinoin (13-*cis*-RA), alone or combined with epigenetic modifiers or low doses of chemotherapy agents (*Kushner* et al – *2006, Hanh* et al – *2008, Zage* – *2018*). More recently combination of immunotherapy agents with 13-*cis*-RA showed encouraging results in improvement of NB patient's survival (*Kushner* et al – *2018*).

Our group previously described that CIN, a positive allosteric modulator of CaSR approved for clinical use (*Nemeth* et al – *1998*), inhibits NB tumour growth in a xenograft mouse model with low endogenous CaSR expression. Treated tumours presented cells with a more differentiated phenotype and a higher fibrosis induction. In addition, NB cell lines where CaSR was overexpressed, CIN induced caspase-mediated apoptosis (*Rodríguez-Hernández* et al – *2016*). In spite of the encouraging results of this study, there are still some side effects that limit the use of CIN in the treatment of NB. The first is related with the low-to-absent expression of CaSR in high-risk NB tumours (*de Torres* et al – *2009*). In addition, we reported that the anti-tumorigenic potential of CIN is seen after prolonged exposure to the drug. More importantly, CIN activates the CaSR present in the parathyroid glands to inhibit plasma PTH secretion and reduce plasma $Ca^{2+}$ concentration, which can have harmful effects in already debilitated NB patients. This work is focused on addressing these challenges in targeting CaSR in NB. First, we were interested in increasing the efficiency of CIN by combining it with drugs that could induce an increase in CaSR expression levels. Next, we aimed to identify a new calcimimetic with potential specific NB anti-tumorigenic properties without inducing hypocalcemia.

### 10.1 Anti-tumorigenic potential of CIN is not increased by combination with 1,25-D$_3$

Autosomal dominant hypocalcemia (ADH), a disease caused by a highly active CaSR leading to low plasma Ca$^{2+}$, is treated with the active metabolite of the vitamin D, 1,25-D$_3$, in combination with dietary Ca$^{2+}$ intake or Ca$^{2+}$ supplements (*Hannan* et al – *2019*). 1,25-D$_3$, participates in the regulation of Ca$^{2+}$ and PO$_4^{3-}$, regulating their intestinal absorption, renal excretion and calcium bone mobilization (*Xue* et *Fleet* – *2009*). This treatment strategy should also be used in NB patients to manage the hypocalcemia induced by CIN treatment. Moreover, 1,25-D$_3$ actively contributes to the control of proliferation and differentiation in a variety of normal and malignant cells (*Deeb* et al – *2007*). In NB, the anti-proliferative and differentiating effects of 1,25-D$_3$ were also reported (*Moore* et al – *1996; Veenstra* et *al* – *1997).* Additionally, *CaSR* gene presents VDREs in both promoters and treatment of colon cancer cell lines, a tumour in which CaSR is epigenetically repressed, upregulates mRNA and protein expression levels of this GPCR (*Fetahu* et al – 2014). We hypothesised that treatment with 1,25-D$_3$ could increase CaSR expression in NB, thus potentiate the anti-tumorigenic effect of CIN.

Our data showed that 1,25-D$_3$ induced an increase of the mRNA levels of *CaSR* in LA-N-1 and IMR5 cell lines at different timepoints and doses. However, expression of this GPCR was highly variable between assays, as mRNA levels are at the detection limit in both cell lines. An earlier study from our group showed that although *CaSR* promoters in LA-N-1 cells were highly methylated, low levels of CaSR protein can be detected. However, promoter hypermethylation was also present in other NB cell lines where *CaSR* mRNA was not detected (*Casalà* et al – *2012*).

GPCR biosynthesis is carried out in the endoplasmic reticulum (ER). Throughout the translation process, this class of receptors is subjected to glycosylation in one or more asparagines and they are often stabilized by disulphide bonds in cysteines in the extracellular loops 1-2 (*Bulenger* et al – *2005*). Similarly, CaSR exhibits a large extracellular domain with several glycosylation sites which undergoes several quality controls during translation (*Cavanaugh* et al – *2010, Zhang* et al – *2016*). Even though we were able to detect the presence of the glycosylated and the non-glycosylated forms in NB cells, as the expression of this

receptor is very close to the detection range. Thus, we were unable to determine the effect that 1,25-D$_3$ had in the expression of the CaSR functional dimer.

Inhibition of cell viability of six different NB cell lines was reported when treated with 1,25-D$_3$ or it analogue 20-*epi*-1,25-D$_3$. In addition, these two vitamin D metabolites inhibited colony formation in a soft agar assay (*Gumireddy* et al – *2003*). Conversely, our results showed that 1,25-D$_3$ as a single agent showed no effect in the proliferation or colony capacity formation of LA-N-1 or IMR5 cell lines. We have to consider that in the previous study authors used a concentration of 1,25-D$_3$ ten times the concentration we used. On the other hand, low expression of CaSR in these cell lines prevents a strong effect of CIN in altering the behaviour of IMR5 and LA-N-1, since the effect induced by this calcimimetic in the proliferation of NB cell lines was determined in cells overexpressing this receptor (*Rodríguez-Hernández* et al – *2016*).

Neural crest cells show the ability to differentiate into several types of cells by the action of intra- and extracellular stimuli expressed at different time points (*Sauka-Spengler* et *Bronner-Fraser* – *2008*). NB is thought to originate from an embryonic failure of neural crest cells and NB cell lines maintain the ability of differentiate into glial and neuronal cells (*Ratner* et al – *2016*). To evaluate the effect of a drug in cell differentiation it is necessary to quantify the expression of many genes associated with NB differentiation (*Chaudhari* et al – *2017*). Our data shows that prolonged treatment of two neuroblastoma cell lines with 1,25-D$_3$ alone or in combination with CIN had divergent effects in each cell line. It was surprising to observe a reduction in the protein levels of the differentiation marker TrkA in LA-N-1 cells treated with 1,25-D$_3$, suggesting a more aggressive behaviour, as there was also an increase of the stem marker *Snail2* (*Taneyhill et al* – *2007*). Moreover, protein levels of NF68 were reduced in cells treated with CIN and this reduction was stronger in the combination treatment. Protein data differs from mRNA where we found a higher expression of the differentiation markers *TrkA*, *Tubβ3* and *CD44* and a reduction of *Snail2*. This divergence on the expression of differentiation markers might be a result of the level of differentiation induced, as differentiation associated genes are up- and down-regulated at different stages and NF68 is expressed at late stages (*Laser-Azogui* et al – *2015*). The effect of 1,25-D$_3$ had in differentiation of LA-N-1 cells contradicts the results that other authors obtained using the LA-N-5 cell lines, a related cell line. These differences might account, at least partially, for the

use of a different methodology to evaluate cell differentiation, such as an increase in neurite outgrowth, acetylcholinesterase activity and decreased invasiveness (*Moore et al – 1995*). In studies conducted using IMR5 there was no evident effect of any drug in mRNA expression levels; however, our data show a more differentiated morphology in cell treated with the combination of CIN and 1,25-D$_3$. Also, and in line with the observed phenotype changes, there was an upregulation of TrkA and NF68 protein levels. This effect on cell differentiation agrees with the reduced invasiveness observed in this cell line by Gumireddy, but it diverges from the results obtained with LA-N-1 (*Gumireddy* et al – *2003*). The differences observed can be explained partially, for the different endogenous expression levels of VDR in both cell lines, being more than six times higher in IMR5. In addition, unlike LA-N-1, IMR5 cells are devoid of *TP53* mutation, tumour suppressor gene that was found to directly upregulate VDR and collaborate with in tumour suppressive functions (*Maruyama* et al – *2006; Reichrath* et al – *2014*).

Taken together, our *in vitro* data show that treatment with 1,25-D$_3$ increases *CaSR* expression in NB cell lines. However, the effect induced by 1,25-D$_3$ does not potentiate the anti-tumorigenic properties of CIN in NB.

## 10.2 Anti-tumorigenic capacity of retinoids is not exacerbated with CIN combination

For decades, retinoids were established as potential anti-cancer agents due to their differentiation capacity and ability to regulate cell growth. Many studies demonstrated the role of retinoids (ATRA, 9-*cis*-RA, 13-*cis*-RA, fenretinide) in the treatment of several types of cancer (*Altucci* et *Gronmeyer* – *2001*). The most effective clinical use of ATRA was demonstrated in the treatment of acute myeloid leukemia (APL). In NB, 13-*cis*-RA is administrated as a part of the treatment schedule for high-risk patients (*Stahl* et *Tallman* – *2019; Reynolds* et al – 2003). However, this promising therapy has some limitations due to retinoid toxicity and resistance observed in many patients. Although ATRA shows a stronger differentiation capacity than 13-*cis*-RA, this latter isomer was found to have a better pharmacokinetics and caused less side effects in children (*Reynolds* et al – *2003*). Our group previously reported that CaSR expression is higher in more differentiated NB and that CIN inhibits NB tumour growth by promoting differentiation *(Rodríguez-Hernández* et al – *2016)*. Thus, we hypothesised that the combination with ATRA

could increase the efficiency of CIN by promoting CaSR expression and cell differentiation

Our data showed that both retinoids were able to increase CaSR mRNA and protein expression levels in LA-N-1 and IMR5 cell lines. Although there was a high variability between replicates, retinoids induced an upregulation of *CaSR* mRNA levels. As we previously published, ATRA was a strong inducer of *CaSR* (*Casalà* et al – *2012*), and our data demonstrate that 13-*cis*-RA consistently increased *CaSR* mRNA after 48-hour treatment. However, CaSR protein detection is very challenging due to the low quality of the commercially available antibodies and the ADD clone antibody was found to be the most specific and reliable antibody for the detection CaSR (*Graca* et al – *2016*). Thus, low expression of this GPCR in NB cell lines prevents us, at least partially, to make a reliable and formed conclusion about the protein results obtained when cells were treated with retinoids.

Differentiation of NB cell lines induced by retinoids occurs concomitantly with cell cycle arrest and consequent reduction in cell proliferation (*Qiao* et al – *2014*). In NB, it was found that retinoic acid induces cell cycle arrest via inhibition of cyclin kinases (*Wainwright* et al – *2001*). In addition, we previously showed that CIN reduces the viability of NB cells that overexpress CaSR (*Rodríguez-Hernández* et al – *2016*). In an attempt to replicate these results in a more endogenous manner, we treated LA-N-1 and IMR5 cells with ATRA or 13-*cis*-RA to increase CaSR expression previous treatment with CIN. LA-N-1 viability was significantly reduced when cells were treated with retinoids, but this effect was not significantly exacerbated by addition of CIN. Surprisingly, IMR5 cells were not as affected by retinoid single treatment. However, and more importantly, ATRA potentiated the inhibition of cell proliferation induced by CIN. In line with our data, Voigt and colleagues showed that ATRA and 13-*cis*-RA inhibited the proliferation and cell adhesion of ten different cell lines. In this study it was also stated that treated cells associated with the S-type phenotype were more sensitive to retinoids. (*Voigt* et al – *2000*). Response to retinoids was more evident in the colony formation assay where little to no colonies were formed in the wells treated with retinoids, suggesting that a prolonged treatment of NB cell lines is more effective than short treatment. Furthermore, this assay evaluates the effect that drugs have in cell line stemness capacity, which was highly reduced when NB cell lines were treated with retinoids (*Rajendran* et *Jain* –

*2018*). These results replicated the first studies that demonstrated the differentiation effect of retinoic acid in NB cell lines (*Sidell – 1982*).

On the other hand, both LA-N-1 and IMR5 showed morphological features corresponding with a more differentiated status when treated with retinoids. Accordingly, LA-N-1 cells treated with retinoids increased the expression of genes associated with NB differentiation, such as *NEFL* and *TrKC*, as well as *CaSR*. As expected, based on the viability assays, retinoids did not potentiate the effect in cell differentiation induced by CIN in LA-N-1 cell line., Prolonged exposure to CIN as a single agent was not as effective inducing differentiation, as we previously published (*Rodríguez-Hernández* et al – *2016*) Recently, it was published that PDX cells cultured *in vitro* in the presence of FBS grown in monolayer and start to differentiate (*Persson* et al – *2017*). As NB cell lines were established from high-risk NB tumours, this work makes us think that cell lines grown for several passages in the presence of FBS might have a more differentiated status. As LA-N-1 cell line is in our laboratory for a long time, its differentiation status increased with the years in the lab, being less sensitive to the treatment with CIN.

It has been described that 13-*cis*-RA did not induce neuronal differentiation of IMR5 cells, as 13-*cis*-RA reduced mRNA levels of *TrkA* and *CHD5*, and no neurite outgrowth (*Higashi* et al – *2015*). Our results diverge from these, as IMR5 cells showed a more differentiated morphology when treated with ATRA or 13-*cis*-RA. In line with the work of Higashi, we saw a reduction in the expression of *TrkA* and *CHD5.* However, our results suggest that 13-*cis*-RA could induce differentiation by increasing of different differentiation markers as *Tubβ3*. On the other hand, 13-*cis*-RA increased the expression *TGM2*, a direct target of the RAR-RXR complex (*Tee* et al – *2010*). Although our results have similarities with Higashi, our main conclusion differs as most of our data supports positive response by IMR5 to retinoids. In IMR5 cells, CIN induced a mildly differentiated morphology and an increase in the expression of *CaSR* and *Tubβ3*. This demonstrates that in IMR5, CIN alone can induce differentiation, albeit not as strong as retinoids.

Treatment with retinoids shows some limitations since about half of NB patients do not respond, and in recent years, there has been an attempt to understand the resistance observed in these patients (*Zage – 2018*). Neurofibromin-1 (NF-1) was found to be one of the main molecules responsible for retinoid response, as SH-SY5Y cells where NF-1 was knock-down did not respond to ATRA

and continue to proliferate. In the same study it was seen that tumours with a high expression of NF-1 were shown to respond to 13-*cis*-RA therapy and present higher survival than those with low NF-1 expression (*Holzel* et al – *2010*). The same group demonstrated that ZNF423 was essential for retinoic acid differentiation and a marker for good NB prognosis (*Huang* et al – *2009*). Our data showed that *NF-1* and *ZNF423* mRNA levels were reduced in cells treated with 13-*cis*-RA or ATRA suggesting a selection of the more resistant cells, as those with high expression responded to retinoids with cell death, while cells with low expression of these two proteins survived prolong retinoid treatment.

As our principal goal was to potentiate the anti-tumorigenic effect of CIN *in vivo*, we first evaluated the effects of short-term ATRA treatment had in the expression of CaSR in LA-N-1 xenograft models. CaSR mRNA and protein levels as well as the tumour differentiation genes, *TrkA* and *NEFL,* were unaltered by short term administration of ATRA. For this reason, we decided to evaluate the anti-tumorigenic effect of the concomitant administration of CIN and ATRA in the same *in vivo* model. Surprisingly, this combination seemed to have a tumorigenic potential, since treated tumours grew faster than those that received single treatments. When testing the possible combination of CIN with 13-*cis*-RA, we observed that CIN worked better alone, albeit the difference is not statistically significant. One of the problems we faced in our *in vivo* studies was retinoids solubility. These compounds are very hydrophobic, and it has been documented the difficulty in administrating these drugs to patients unable to swallow pills, such as children. This low solubility often resulted in miss-dosages of the drug and reduced plasma concentration (*Takitani* et al – *2004*). Pharmacokinetics of CIN has to be considered when analysing the results of its combination with 13-*cis*-RA, since both were administered orally and simultaneously. In pharmacokinetic studies it was seen that administration with high- or low-fat diet increased drug exposure in comparison with fasting (*Padhi* et *Harris* – *2009*). However, animals in our studies have access to food *ad libitum,* thus comparison with these human studies might be challenging.

Taken together, our *in vitro* and *in vivo* data allowed us to conclude that combination of retinoic acid and CIN did not increase the efficacy of retinoids as single agents.

**10.3 Activation of CaSR with AC-265347 inhibits NB tumour growth while maintaining physiological plasma Ca$^{2+}$ levels**

Activity of GPCRs is regulated by allosteric modulators, organic and inorganic molecules including their natural ligands. In the case of CaSR, the positive or negative allosteric modulators are called calcimimetics or calcilytics, respectively (*Nemeth* et *Goodman* – 2016). Diseases associated with poor regulation of plasma Ca$^{2+}$ levels created the need for the development of synthetic calcimimetics and calcylitics that modulate CaSR in patients suffering from calciotropic diseases (*Hannan* et al – *2019*). The first allosteric modulator to be approved for clinical use was CIN, with the intent of treating patients with hypercalcemia associated with parathyroid cancer, primary hyperparathyroidism (HPT) not candidate to parathyroidectomy, and secondary HPT in chronic kidney disease patients undergoing dialysis (*Nemeth* et al – *1998; Nemeth* et *Goodman* – 2016). In our previous publication we proposed to repurpose CIN for the treatment of NB as this calcimimetic showed anti-tumorigenic properties since it inhibited *in vivo* tumour growth by promoting differentiation, ER stress, apoptosis and/or fibrosis (*Rodríguez-Hernández* et al – *2016*). However, one of the handicaps for the use of CIN to treat NB patients is the marked reduction of plasma Ca$^{2+}$ levels induced by this calcimimetic.

To overcome this, we evaluated if AC-265347, a calcimimetic shown to have a mild effect in plasma Ca$^{2+}$ levels and strong effect in PTH inhibition (*Ma* et al – *2011*), shows neuroblastoma anti-tumorigenic effects similar to CIN. It was reported that AC-265347 binds to the transmembrane domain of CaSR and favours intracellular signalling via pERK1/2 over Ca$^{2+}$ mobilization (*Cook* et al – *2015*). According to published data, our results show that AC-265347 did not significantly reduce plasma Ca$^{2+}$ levels of healthy immunocompromised athymic nude mice. More importantly, in a LA-N-1 xenograft model, AC-265347 was able to significantly inhibit tumour growth similar to CIN. Quantification of tumour necrosis showed that tumour treated with CIN slightly showed higher necrosis percentage than those treated with AC-165237 or VEH. On the other hand, there was no difference in amount of fibrosis induced, or the expression levels of the proliferation marker Ki67. These results are in discordance with our published results, were we showed that CIN increased tumour fibrosis (*Rodríguez-Hernández* et al – *2016*). An explanation

for these discrepancies with our previous data could be, at least partially, due to the method used to quantify fibrosis in both studies: in this work we used an automated software that quantified the green staining, specific of fibrotic fibres, present in the whole tumour, while in our previous work several photos of different fields were taken and fibrotic area was quantified by a blind researcher. As expected and based on our previous results, CIN induced an upregulation of CaSR expression. However, this was not statistically significant due to the high variability of expression in VEH treated tumours, since quantification of the whole tumour allows for a more reliable evaluation of the effect, albeit it introduces greater variability within treatment groups.

Despite the fact that both calcimimetics induce a more differentiated phenotype, histological quantification of specific differentiation markers showed that only CIN significantly upregulates TrkA and NF68 expression levels. Surprisingly, tumours treated with AC-265347 significantly expressed higher levels of the differentiation marker CD44. Differential gene expression induced by each drug might be an indicator of differential molecular mechanisms activated by CIN and AC-265347. Both calcimimetics increased the expression of CaSR, corroborating the published data demonstrating the calcimimetics modulate both the expression and activity of CaSR (*Mendoza* et al – *2009, Nemeth* et *Goodman – 2016*). Furthermore, analysis of gene expression levels corroborated histological data, as tumours treated with CIN show significantly upregulation of *NEFL*, *TrkA*, *TUBB3* and *CHD5*. The hypothesis of a possible differential effect of these two calcimimetics gains strength when we observed that the expression of most genes associated with NB differentiation were mildly altered in tumours treated with AC-265347. Overexpression of NFGR was found to increase differentiation of NB cells lines (*Schulte* et al – *2009*), however in our data the two calcimimetics tested decrease the expression of this receptor. This is most likely due to the stage of differentiation that these drugs induce, as different genes are increased and exert its functions at different times of the differentiation process (*Kholodenko* et al – *2018*).

Although not many genes were significantly altered by AC-265347, we observed a significant upregulation of *TGFβ*. Average expression of *TGFβ* was similar in AC-265347 and CIN treated tumours, however high variability between samples prevents statistical significance relative to VEH treated tumours. In hepatic cells and HEK cells, activation of CaSR inhibited TGFβ signalling and expression via Smad (*Organista-Juárez* et al – *2013*). An explanation for this discrepancy with our

data could be that CaSR activation is tissue dependent, and this receptor can present opposite effects depending on cell type (*Hannan* et al – *2019*). Similarly, TGFβ signalling also shows different effects depending on the target tissue (*Morikawa* et al – *2016*). Since TGFβ presents an important role in adult neurogenesis (*He* et al – *2014*), in neuroblastoma, upregulation of TGFβ induced by AC-265347 might be partially, an indicator of a more differentiated phenotype. On the other hand, inhibition of this protein was recently found to be important for retinoid treatment resistance (*Duffy* et al – *2017*), hence it would be interesting to evaluate if the treatment with AC-265347 could potentiate the effect of retinoids.

Recently it has been described that activation of CaSR induced the expression and secretion of anti-inflammatory cytokines in colon cancer cells lines with an over expression of CaSR (*Iamartino* et al – *2020*). Moreover, TGFβ plays a plethora of roles in the immune system cells and shows anti-inflammatory properties (*Sanjabi* et al – *2017*). Thus, it would be interesting to study if these drugs could induce also an anti-inflammatory effect in NB tumours.

According to our previous published data, CIN significantly increased the expression of several cancer-testis antigen (CTA) genes, while AC-265347 treated tumours increased only the expression of some of these genes.. This family of genes codes for several antigens that were found to be important as immunotherapy targets (*Krishnadas* et al – *2013*). The fact that both calcimimetics increase their expression to different extents, makes us think that CIN and AC-265347 might be interesting drugs to combine with immunotherapy agents.

Patient-derived xenograft models (PDX) are more reliable *in vivo* tumour models, as they maintain tumour heterogeneity and show a similar microenvironment to tumours of the patients (*Braekeveldt* et al – *2016; Braekeveldt* et al – *2018*). First, we observed that all PDX models that were generated for our group expressed detectable levels of CaSR. Next, we evaluated the effect of AC-265347 and CIN in the three models with better engraftment. In the three models tested, only HSJD-NB001 responded to the treatment with calcimimetics, where both calcimimetics significantly inhibited tumour growth. These results demonstrate that effect of calcimimetics is model dependent and the lack of response from HSJD-NB005 and HSJD-NB012 might be due to different reasons. First, protein expression of CaSR in the three PDX models varies, showing the highest level the HSJD-NB001 model.

According to our data, high levels of CaSR confer sensibility to calcimimetics treatment. On the other hand, HSJD-NB005 was generated from a very aggressive patient tumour showing a fast growth in mouse models. We previously demonstrated that tumours that respond to CIN need a prolonged exposure to this drug (*Rodríguez-Hernández* et al – *2016*). Moreover, engraftment rate of the HSJD-NB012 model is the lowest of the three models used, hindering the tumour growth. Thereby, we observed a high variability between the two independent experiments. In the first experiment, both calcimimetics significantly inhibited tumour growth; however, in the second experiment, VEH treated tumours had a slower growth rate, and pooling of the two experiments showed no effect of calcimimetics in tumour growth. As the tumours of the second experiment underwent another serial passage, they might have some alteration that alter their response to calcimimetics (*Sato* et al – *2019*). Thus, it would be interesting to evaluate tumorigenesis features of VEH treated tumours from both experiments to understand why they have different growth rates.

Similar to LA-N-1 xenograft model, in HSJD-NB001, CIN induced a more differentiated phenotype accompanied by a significant upregulation of TrkA and NF68 differentiation markers. However, tumours treated with AC-265347 appeared to have an intermediated phenotype, as these markers were increased, albeit there was no statistical significance. The different gene expression pattern observed in both treatments could suggest a different mechanism of action to exert their anti-tumorigenic effect. HSJD-NB001 is a MYCN amplified tumour, and it was encouraging that AC-265347 significantly decreased the mRNA levels of this oncogene. A decrease of expression of *MYCN* is found in retinoic induced neuroblastoma differentiation (*Thiele* et al – *1998*) and might be an indicator of the higher differentiation status of tumours treated with AC-265347. Differently to LA-N-1 xenografts treated with CIN, there was a reduction of the expression of CTAs in HSDJ-NB001 tumours treated with this calcimimetic. This discrepancy might be due to the different genetic alterations in both models, we only know that both are *MYCN* amplified and *TP53* mutated.  These are not the only genetic alterations found in NB tumours and it would be interesting to further study genetic alterations found in LA-N-1 cell and HSJD-NB001 tumours as they might give us an understanding about their different responses to calcimimetics.

In vitro studies reproduced quite notably effects induced by this drug in vivo, becoming evident that CIN and AC-265347 exert their anti-tumorigenic activities via different mechanisms. Cytotoxicity is the main mechanism of chemotherapy drugs, some of which induce an increase in intracellular $Ca^{2+}$ concentrations (*Cui* et al – *2017*). Activation of GPCRs induces $Ca^{2+}$ mobilization via $G\alpha_{q/11}$ and we were interested in knowing if AC-265347 might induce cytoxicity by the same mechanism. The high IC50 values (drug concentration needed to inhibit 50% of the cells) demonstrated that AC-265347 does not exert cytotoxic effects in the five NB cell lines tested. Additionally, we observed that the two NB cell lines used, LA-N-1 and IMR5, responded differently to calcimimetics, both in prolonged and acute exposure assays, being the IMR5 cell line the more sensitive to both treatments. Furthermore, AC-265347 seems to have a stronger effect in IMR5, as cell treated with this drug increase the expression of most NB differentiation genes. In the acute exposure assays in the presence of extreme $Ca^{2+}$ concentrations, both cell lines respond differently. Percentage of apoptotic LA-N-1 cells was higher when they were treated with CIN than with AC-265347 or their vehicle, in IMR5 cells it was the reverse, where treatment with AC-265347 induced more apoptosis than CIN. The main difference in both cell lines is the status of *TP53*, which is mutated in LA-N-1 cells and unaltered in IMR5. This tumour suppressor gene is not often altered in NB; however it is found mutated in relapse and refractory NBs (*Kim* et *Shohet* – *2009, Zage* – *2018*) and alteration of this gene is associated with drug resistance (*Tweddle* et al – *2001*). As *TP53* is important in several pathways, alteration of these gene alters several cellular pathways and it is unclear the effect that this can have in the modulation of CaSR signalling.

It has been described that in HEK293 overexpressing CaSR, these two calcimimetics activate different G-protein subunits leading to the activation of different signalling cascades (*Cook* et al – *2015*). Our results show that this effect was cell line dependent, where it was demonstrated that IMR5 cells were more responsive to calcimimetics. On the other hand, AC-265347 was a better differentiation inducer than CIN. In addition, activation of different pathways by these two calcimimetics has been reported in a HEK-CaSR model, as a result of stabilization of different conformations of CaSR (*Cook* et al – *2015, Leach* et al – *2016*). This different G-protein coupling was shown to be an important aspect in breast cancer development, where CaSR was found to be coupled to $G\alpha_{i/o}$ in

mammary tissue and to Gα$_s$ in breast cancer cell lines (*Mamillapalli* et al – *2010*). In the nervous system, CaSR was found to be important for neurodevelopment as well as in the onset of Alzheimer's disease and ischemia (*Giudice* et al – *2019*). Future studies should attempt to understand the different pathways activated by CIN and AC-265347 and what are the anti-tumorigenic processes triggered.
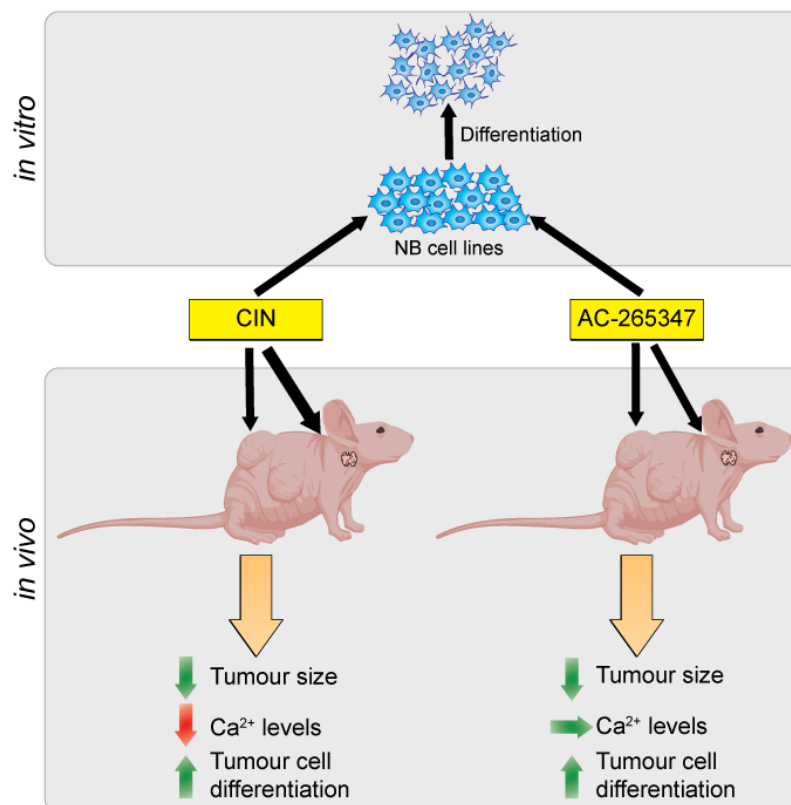


**Figure 37 – Tumour growth of NB is inhibited by activation of CaSR by two different calcimimetics.** Activation of CaSR present in tumour cells and parathyroid glands can have different effects, here we demonstrate that AC-265347 inhibits tumour growth and has a mild effect in plasma Ca$^{2+}$ levels, contrary to CIN that has a strong effect in reducing both tumour growth and plasma Ca$^{2+}$ levels.

Taken together, we identified a new calcimimetic with potential specific NB anti-tumorigenic properties without inducing hypocalcaemia (Figure 37). Differentiation capacity of this calcimimetic could make it an interesting approach, alone or in combination with other epigenetic modifiers or low doses of chemotherapy agents, to control minimal residual disease and to prevent relapses in NB.

# VI.  Conclusion

# 11    Conclusion

## Conclusions

In this thesis, we describe different strategies to target CaSR as a new potential therapeutic approach for the treatment of neuroblastoma. We perform different *in vitro* and *in vivo* assays to evaluate how to increase the efficacy of CIN or find alternatives to the administration of this calcimimetic in the treatment of neuroblastoma. The main conclusions related to work are:

1)    Active from of vitamin D, 1,25-D3, increases the expression of *CaSR* in two NB cell lines with low endogenous expression of this GPCR.

2)    Anti-tumorigenic effect of CIN is not potentiated by the combination with 1,25-$D_3$. This treatment does not affect cell viability, clonogenic capacity or differentiation status of NB cell lines.

3)    Retinoids are strong inducers of CaSR expression.

4)    Both retinoids used in our studies, ATRA and 13-*cis*-RA, induce a strong differentiation effect in NB cell lines. However, their effect is not increased by the combination with CIN.

5)    Using a xenograft mouse model, we show that single treatment with retinoids or CIN delay neuroblastoma tumour growth. However, we do not observe any additive effect when the drugs were combined.

6)    CIN and AC-265347 inhibit neuroblastoma tumour growth in two neuroblastoma tumour models. Moreover, AC-265347 does not alter in plasma $Ca^{2+}$ levels.

7)    We show that CIN induced an increase of NB differentiation markers in both *in vivo* tumour models, while the effect of AC-265347 in the same genes was seen only in the PDX model. This differential effect of these two calcimimetics suggests the activation of different mechanisms in the inhibition of tumour growth.

8)    *In vitro* assays showed a mild effect of calcimimetics in apoptosis induction. However, prolonged exposure of two NB cell lines to AC-265347 and CIN, revealed an alteration in the expression of NB differentiation markers after treatment. This calcimimetic differential effect is more evident in IMR5 cells, where AC-265347 was a better inducer of differentiation than CIN.

# VII. Bibliography

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# VIII.  Annexes

## Annex I – List of primers

| Gene | Assays on Demand | Primer Forward | Primer reverse |
|---|---|---|---|
| ADCY8 | | CGCCAAGCTTTCCTGGAGAC | ACGAGCCGCTCCTGTCTTTG |
| ALDH1A2 | | ACTAATGACATCAACAAGGC | CCAAATTCTCCCATTTCTCT |
| ANXA8 | | ACTCTACTATGCCATGAAGG | ATTTAAGTCAATCTCGCTCC |
| ATF4 | | GTGGCCAAGCACTTCAAACC | CCCGGAGAAGGCATCCTC |
| BID | | CTTAGCCAGAAATGGGATG | AGTCACAGCTATCTTCCAG |
| CaSR | Hs01047795_m1 | | |
| CD44 | Hs01075861_m1 | | |
| CDH5 | | CCACAACGAGGGCATCATCA | GAGGTCGATGGTGGGGTCTG |
| CHOP | Hs99999172_m1 | | |
| COL3 | | TGATGGTGTCCCAGGGAAAGA | GGGCACCACCTTCACCCTTA |
| COL1A | | ATGTCTGGTTCGGCGAGAGC | CGCAGGAAGGTCAGCTGGAT |
| CTCFL | | GCACGGAAAAGCGACCTACG | TGAGGGCATAGCGTTCATGG |
| CTGF | | TTGCGAAGCTGACCTGGAAGAGAA | AGCTCGGTATGTCTTCATGCTGGT |
| EGF | | TTGCACAACCCAAGGCAGAA | TGCTGCTGCAGTTTCCTTTCC |
| EGFR | | GGTGGTCCTTGGGAATTTGGA | CAGCCACCTCCTGGATGGTCTT |
| GAGE-P1 | | GCAGCTGCTCAGGAGGGAGA | TGTGGGTGACCCTGTTCCTG |
| GAGE-P2 | | GGCCGAAGCCTGAAGCTCAT | ATTTGGCGGGTCCATCTCCT |
| GAGE-P3 | | GAGGGAGCATCTGCAGGTCAA | CCTGCCCATCAGGACCATCT |
| GRP78 | | GGCCGAGGAGGAGGACAAG | ACGCGGCCGTTCTTGAACAC |
| ID2 | | TCCTGTCCTTGCAGGCTTCTG | TCAGCCACACAGTGCTTTGC |
| MAGE-A2 | | AACCAGGCAGTGAGGCCTTG | AGCCTGTCCCCCTCAGAACC |
| MAGE-A3 | | AAGGTGGCCGAGTTGGTTCA | ACTGCCAATTTCCGACGACA |
| MAGE-A6 | | CGGGGATCCCAAGAAGCTG | TTTCAATGAGGGCCCTTGGA |
| MMP2 | | CAAGGAGTACAACAGCTGCACTGATA | GGTGCAGCTCTCTCATATTTGTTGC |
| MYCN | Hs00232074_m1 | | |
| NF1 | | TGTCAGTGCATAACCTCTTGC | AGTGCCATCACTCTTTTCTGAAG |
| NEFL | Hs00196245_m1 | | |
| NOXA | | CTACAATGGTGATAACTGTGAG | CAGTCATCCTCATTAATCTCG |

| | | | |
|---|---|---|---|
| **TrkA** | Hs01021011_m1 | GACGCTTCGTGTTCAGCTCGCG | CTCGGTTGAGCGTTCTTGC |
| **TrkC** | Hs00176797_m1 | | |
| **NY-ESO-1** | | TGCTTGAGTTCTACCTCGCCAT | GCTCCTGCGGGCCAG |
| **NGFR** | Hs00609977_m1 | | |
| **PRKCA** | | CTGAGAAGAGGGGGCGGATT | GGATCTGAAAGCCCGTTTGG |
| **PUMA** | | ACGACCTCAACGCACAGTACGAG | AGGAGTCCGCATCTCCGTCAGTG |
| **RARα** | Hs00940446_m1 | | |
| **RARγ** | Hs01559234_m1 | | |
| **RXRα** | Hs01067640_m1 | | |
| **RYR** | | GCTCAAGGTGGTGGTCATCG | TTGAAGACCGGGAGGTGGAC |
| **RYR2** | | GCTCAAGGTGGTGGTCATCG | TTGAAGACCGGGAGGTGGAC |
| **S100-β** | Hs00902901_m1 | | |
| **Snail1** | Hs00195591_m1 | | |
| **Snail2** | Hs0 0950344_m1 | | |
| **SSX4/4B** | | CACCCTCCCACCTTTCATGC | GGACGTTCAACCTGATTCCTGTG |
| **TBP** | | GAACATCATGGATCAGAACAACAG | ATTGGTGTTCTGAATAGGCTGTG |
| **TERT** | | TGTCTGGAGCAAGTTGCAAAGC | GCTGCCTGACCTCTGCTTCC |
| **TGFβ1** | | CTGGAGAGGGCCCAGCATCT | CGCACGCAGCAGTTCTTCTC |
| **TGFβ2** | | CCTTCTTCCCCTCCGAAAC | AGAGCACCTGGGACTGTCTG |
| **TGFβ3** | | TGCCCAACCCCAGCTCTAAG | CGCTGTTTGGCAATGTGCTC |
| **TGFβR3** | | TTCCCTGTTCACCCGACCTG | GATGTTTCCGTGGGGCTGTT |
| **TGM2** | | CAACCTGGAGCCTTTCTCTG | GCACCTTGATGAGGTTGGAC |
| **TRB3** | | TGCTCCAGATCGTGCAACTG | GGTACCAGCCAGGACCTCAG |
| **Tubβ3** | Hs00801390_m1 | | |
| **TWIST1** | | AAGGCATCACTATGGACTTTCTCT | GCCAGTTTGATCCCAGTATTTT |
| **VCAN** | | GCAGCTCTTTGCTGCCTATGAA | GAGCCCGGATGGGATATCTG |
| **VEGF** | | GCCCACTGAGGAGTCCAACA | TCCTATGTGCTGGCCTTGGT |
| **VIM** | | TACAGGAAGCTGCTGGAAGG | ACCAGAGGGAGTGAATCCAG |
| **ZNF423** | | TGACGACCCACAACTCTCCT | GAACTGGCAAGGGTATGGCAG |

## Annex II – Curriculum Vitae

## Eliana Carolina Goncalves Alves, MSc

**Telephone:** +34 622460843 or +352 917872584

**email:** eliana.alves663@gmail.com

**Nationality**: Portuguese

**Date of birth**: 9th of October 1989

### Professional experience and Education

**2016 – Present:** <u>PhD in Biomedicine</u> from the University of Barcelona. Work elaborated in the Department of Developmental Tumours from the Fundació Sant Joan de Déu, Barcelona, Spain.

Dissertation: "Combined therapies for neuroblastoma based on the calcium sensing receptor (CaSR)"

Supervisors: Dr. Carmen de Torres, PhD, Silvia Mateo-Lozano, PhD and Cinzia Lavarino, PhD

**2016 – 2019:** <u>Early Stage Researcher</u> in the CaSR Biomedicine Inovative Training Network (Marie Skłodowska-Curie Actions) in the project entitled "Evaluation of CaSR targeted drugs for the treatment of neuroblastoma"

Principal investigator: Dr. Carmen de Torres, PhD

- Use of preclinical cancer models to evaluate anti-tumorigenic capacity of drugs targeting CaSR
- Management and development of research project
- Presentation of results in international training network meetings
- Establishing and developing international relationships

**2011 – 2015:** <u>Research Assistant</u> in the Department of Rheumatology at the Medical University of Vienna.

Principal investigator: Ass.-Prof.  Priv.Doz. Dr.med.univ. Stephan Blüml

- Management of transgenic mouse colonies
- Planning and preforming autoimmune preclinical studies
- Training of phD and visiting students in animal facility practices

**2013 – 2015:** <u>Master's in Molecular Biology</u> (specialization Molecular Medicine) from the University of Vienna.

Dissertation: "T helper cell activation and clonal expansion after antigen presentation is regulated by miR-155"

Supervised by: Ao.Univ.-Prof. Dr. Günter Steiner and Ass.-Prof. Priv.Doz. Dr.med.univ. Stephan Blüml

Grade: 1.6 (1-5 system, 1 is the highest grade)

Honors/Awards: Best Master thesis from the Austrian Society of Rheumatism (ÖGR)

**2010 – 2011:** <u>Intern</u> at the Biomed zet Life Science GmbH via the Lifelong Learning Program Leonardo DaVinci.

- Establishing preclinical toxicity tests
- Evaluation of efficacy of toxicity assays

**2007 – 2010:** <u>Bachelor's degree in Applied Biology</u> at the Biology Department in the University of Minho.

Grade: 13 (out of 20)

**Technical Skills**

**Molecular Biology techniques**: Flow cytometry, quantitative real-time PCR (qPCR), PCR, RNA and DNA isolation and quantification, Immunohistochemistry (IHC), Immunofluorescence (IF), ELISA, basic in confocal microscopy

**Preclinical cellular assays**: maintenance, growth and differentiation cell lines; isolation and maintenance of primary immune cells, co-culture of immune cells; differentiation of dendritic cells and macrophages from mouse bone marrow cells; cytotoxic assays;

**Preclinical animal models**: cell line-derived xenograft models, patient-derived xenograft models, collagen induced arthritis (CIA), serum induced arthritis, hTNF transgenic arthritis,

**Laboratory animal handling**: drug administration (Subcutaneous, oral gavage, intraperitoneal, intravenous); tail-vein blood collection; organ and tissue collection

**Statistical analysis**: GraphPad Prism, Microsoft Excel

**Flow cytometry analysis software:** FlowJo, BD FACS Diva, NovoCyte Express

**Image analysis and edition**: Adobe Illustrator, Microsoft PowerPoint, I-view, Image J, STRATA Quest

## Honours and Awards

**2016 ÖGR** (Österreicher Gesellschaft für Rheumatologie) **Diplomarbeitspreise** (1000€) – Price for the best master thesis of 2015

**2014 ÖGR poster prize** – "The role of microRNA-155 in rheumatoid arthritis"

## List of publications

García M, Rodríguez-Hernández CJ, Mateo-Lozano S, Pérez-Jaume S, **Gonçalves-Alves E**, Lavarino C, Mora J, de Torres C; "Parathyroid hormone-like hormone plays a dual role in neuroblastoma depending on PTH1R expression"; Molecular Oncology (2019)

**Goncalves-Alves E**, Saferding V, Schliehe C, Benson R, Kurowska-Stolarska M, Brunner JS, Puchner A, Podesser BK, Smolen JS, Redlich K, Bonelli M, Brewer J, Bergthaler A, Steiner G, Blüml S; "MicroRNA-155 Controls T Helper Cell Activation During Viral Infection"; Frontiers Immunology (2019)

Puchner A, Saferding V, Bonelli M, Mikami Y, Hofmann M, Brunner JS, Caldera M, **Goncalves-Alves E**, Binder NB, Fischer A, Simader E, Steiner CW, Leiss H, Hayer S, Niederreiter B, Karonitsch T, Koenders MI, Podesser BK, O'Shea JJ, Menche J, Smolen JS, Redlich K, Blüml S; "Non-classical monocytes as mediators of tissue destruction in arthritis"; Annals of Rheumatoid Diseases (2018)

Saferding V, Puchner A, **Goncalves-Alves E**, Hofmann M, Bonelli M, Brunner JS, Sahin E, Niederreiter B, Hayer S, Kiener HP, Einwallner E, Nehmar R, Carapito R, Georgel P, Koenders MI, Boldin M, Schabbauer G, Kurowska-Stolarska M, Steiner G, Smolen JS, Redlich K, Blüml S.; "MicroRNA-146a governs fibroblast activation and joint pathology in arthritis"; Journal od Autoimmunity (2017)

Blüml S, Sahin E, Saferding V, **Goncalves-Alves E**, Hainzl E, Niederreiter B, Hladik A, Lohmeyer T, Brunner JS, Bonelli M, Koenders MI, van den Berg WB, Superti-Furga G, Smolen JS, Schabbauer G, Redlich K.; "Phosphatase and tensin

homolog (PTEN) in antigen- presenting cells controls Th17-mediated autoimmune arthritis", Arthritis Res Ther. (2015)

Saferding V, **Goncalves-Alves E**, Blüml S; "MicroRNAs and other non-coding RNAs in inflammation: The importance of microRNAs in Rheumatoid arthritis", Springer 2014 – Book Chapter

## **Others**

- **Languages**
    - o Portuguese – Native
    - o English – C1 Certificate in Advance English (CAE) by the University of Cambridge (2015). Grade B (Good)
    - o German – B2 certificate from the Österreisches Sprachdiplom Deutsch (2013)
    - o Spanish – B2 (Independent User)
- **Executive positions**
    - o Representative of Early Stage Researchers (ESRs) in steering board meetings from the CaSR biomedicine Training Network
    - o Treasurer for the Student Association of Applied Biology of Minho University (2009-2010)
    - o Member of the Logistics department for the organization of the Applied Biology conference, *XI Jornadas de Biologia Aplicada*, 2010
- **Volunteer Activities**
    - o Monitor in the SUPerando association, teaching disable children Stand-up paddle and surf
    - o Delegation host for the Portuguese delegation in the 2015 Eurovision Song Contest, assistance of delegation with schedule keeping and translation.