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"Development of a neuronal cellular model for the study of neurodegenerative diseases"

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ABSTRACT

RATIONAL OF THE STUDY – Neurodegenerative diseases (NDDs) represent a major global health challenge characterized by progressive neuronal loss, synaptic dysfunction, and accumulation of misfolded proteins. Translating novel techniques into effective therapies remains difficult due to evolutionary differences between human and rodent nervous systems, complicating the evaluation of therapeutic efficacy and emphasizing the need for human cell-based models. Urine-derived stem cells (USCs) are particularly valuable because they are patient-specific, cost-effective, and easily accessible via non-invasive collection. Unlike fully induced pluripotent stem cells (iPSCs), USCs retain aspects of the donor's molecular identity, including key epigenetic signatures, age-related marks, and disease-associated traits, allowing more accurate in vitro modelling of disease.

PLANNING OF THE STUDY – This study aimed to establish a reliable neuronal model from healthy human USCs to investigate NDD-related molecular mechanisms. USCs were isolated and differentiated into neurons using two approaches: chemical small-molecule induction and NGN2-mediated lentiviral transduction. The efficiency, maturation, and stability of neuronal differentiation were assessed using phase-contrast morphological tracking, quantitative RT-PCR (qRT-PCR) for key neuronal markers (MAP2, TUBB3, TAU, NCAM, CNTN1, NES, PAX6, NGN2), and immunofluorescence (IF) assays targeting neurogenic proteins (MAP2, DCX, NES).

RESULTS – Small-molecule induction successfully initiated early neuronal commitment, with qRT-PCR showing progressive upregulation of mature neuronal markers (MAP2, TAU, TUBB3) by day 14. However, cell viability dropped sharply after day 11, limiting long-term morphological and protein-level analyses. In contrast, NGN2 lentiviral transduction produced a highly stable, viable neuronal phenotype lasting at least 21 days. Morphological analysis revealed maturation of neurite-like processes by day 7, progressing to complex, interconnected network-like structures by day 21. Molecular analyses reflected this maturation: early progenitor markers DCX and NES peaked at day 7 and decreased thereafter, while mature neuronal markers (MAP2, TUBB3, TAU, NCAM, CNTN1) exhibited robust, persistent upregulation throughout 21 days. The comparison indicates that while small-molecule induction initiates differentiation, NGN2-mediated transduction is more effective and reproducible for generating USC-derived neurons.

CONCLUSIONS – The optimized NGN2-based approach provides a robust, patient-specific in vitro neuronal platform, bridging primary cellular biology and translational neuroscience. This model holds promise for personalized medicine, non-invasive biomarker validation, and mechanistic studies of neurodegenerative pathology.

1. INTRODUCTION

Neurodegenerative diseases (NDDs) are disorders that cause the gradual failure and death of cells in the brain and spinal cord (Menéndez-González M, 2023). Neurodegenerative disorders include a heterogeneous group of conditions that differ substantially in their clinical manifestations, genetic determinants, and pathological features. Selective neuronal vulnerability (SNV) is one of the most obvious but poorly understood features of neurodegenerative diseases, in which subtypes of neurons become pathologized, and others are protected against the deleterious effects. Evidenced beyond the direct consequences of protein aggregation, research indicates that such vulnerability is indeed attributable not so much to protein aggregation but to the interaction between cell-autonomous features and non-cell-autonomous influences at an intricate interface of cell-autonomous and non-cell-autonomous processes. For example, SNc and entorhinal cortical neurons display high metabolic load, large axonal arborization, and sustained pacemaking activity that converge to the elevation of mitochondrial oxidative stress and calcium imbalance (Rodriguez G, 2020; Surmeier, D., 2017). Though the so-called prion-like propagation of misfolded proteins, e.g. alpha-synuclein and tau, across synaptic networks or tunnelling nanotubes points to a systemic progression, recent data indicate that inherent cellular attributes are the determinants for the clearance by and failure in a neuron (Subramaniam, S., 2019; Fu, 2018). In addition, next-generation single-cell transcriptomics and CRISPR-based screens are starting to uncover the so-called molecular 'signatures' of vulnerability, elucidating that the disruption of proteostasis and mitophagy is usually specific to certain genomic profiles (Kampmann, M., 2024). The elucidation of SNV progression from regional anatomic observation to molecular mechanism is salient, as that transition provides a mechanism through which therapeutics can be directed towards improving the resilience particularly of high-risk neuronal subtypes rather than the global clearance of proteins. By selecting from these early neuromodulatory nodes, it may be possible to halt the classic progression of neurodegeneration before a more extensive loss of cognitive and motor circuits can occur. Despite this diversity, they share fundamental mechanisms characterized by selective neuronal loss, synaptic network disruption, and progressive neurodegeneration, processes that are associated with the accumulation of misfolded or aggregated proteins within neurons and glial cells (Forrest et al., 2025). These disorders impose a profound burden on patients, their families, and healthcare systems worldwide, representing a growing public health and socioeconomic challenge. However, therapeutic success in modifying or halting disease progression remains limited. One major contributing factor is that neurodegenerative

diseases are still largely classified based on observable clinical phenotypes rather than on underlying molecular or biological endophenotypes. This phenotypic approach can result in heterogeneous and poorly stratified patient populations in clinical trials, thereby complicating the evaluation of therapeutic efficacy and hindering the development of precision medicine strategies (Menéndez-González M, 2023). Neurodegenerative diseases are generally identified based on several key histological characteristics:

1. the pattern and extent of neuronal loss in the brain, often accompanied by activation of astroglial and microglial cells, correlate with clinical symptoms.
2. the presence of intracellular or extracellular protein aggregates, detectable using antibodies targeting pathological forms of these proteins.
3. an absence of significant infiltration by peripheral inflammatory or cancerous cells, or by infectious agents that could explain neuronal death.
4. no evidence that environmental toxins contribute to the loss of neurons (Forrest S.L et al., 2025).

The most common forms include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), Frontotemporal dementia (FTD), Multiple system atrophy (MSA), Progressive supranuclear palsy (PSP), Corticobasal degeneration (CBD), Spinocerebellar ataxias (SCAs), and Lewy body dementia (LBD). Alongside these major types, several rarer inherited or metabolic conditions are also classified as neurodegenerative, including prion diseases such as Creutzfeldt–Jakob disease, Friedreich's ataxia, spinal muscular atrophy (SMA), hereditary spastic paraplegia (HSP), Pelizaeus–Merzbacher disease, Alexander disease, Canavan disease, metachromatic leukodystrophy, and neuronal ceroid lipofuscinoses (NCLs). However, because of the wide diversity and complexity of these disorders, it is not possible to cover all of them in detail here.

According to the Global Burden of Disease (GBD) 2021 study, the incidence and societal burden of these disorders have continued to grow, largely driven by population aging and extended life expectancy. In sum, these data highlight the urgent need for integrated, multidisciplinary approaches that bridge clinical management, biomedical research, and health policy. Such strategies are essential to mitigate the escalating burden of neurodegenerative diseases and to enhance the overall quality of life of patients and their caregivers.

In the context of molecular genetics and genetic epidemiology, disease-associated genes are broadly categorized into two main classes: causative genes, which directly determine the onset of a pathological condition, and susceptibility genes, which modulate an individual's predisposition to develop the disease. The identification and characterization of these genetic determinants in neurological disorders, together with the implementation of advanced transgenic models, have considerably deepened our understanding of the molecular pathways involved in neurodegeneration. These advances not only elucidate the biological foundations of neuronal dysfunction but also provide critical insights for the development of precision-based therapeutic interventions aimed at mitigating disease progression (Firdaus, 2024). Building on these solid advances, examining specific examples of neurodegenerative disorders and their genetic backgrounds can provide a clearer understanding of the roles played by causative and susceptibility genes. Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease illustrate the complex interplay between causative and susceptibility genes in shaping disease onset and progression. In addition to inherited genetic factors, environmental influences exert a profound impact on the development and course of neurodegenerative disorders. Chief among these is oxidative stress, which arises when the generation of reactive oxygen species (ROS) surpasses the protective capacity of endogenous antioxidant systems. The brain is particularly susceptible to such disturbances, given its high metabolic rate and the abundance of polyunsaturated fatty acids within neuronal membranes. Clinical investigations lend weight to these observations, consistently reporting elevated concentrations of oxidative biomarkers such as malondialdehyde and protein carbonyls together with impaired antioxidant enzyme activity in patients with Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Taken together, these findings underscore the intricate interplay between environmental exposures, oxidative damage, and inflammatory processes, and highlight their central role in the pathogenesis of neurodegeneration (Chandra Dash U, 2024; Mihai Teleanu D, 2022).

Biomarkers are molecules that can be used to observe changes in an individual's biochemical or medical status and provide information to aid diagnosis or treatment decisions. Initially, blood biomarkers for neurodegenerative diseases were considered unreliable due to low sensitivity and poor reproducibility of early assays. With the advent of ultrasensitive technologies such as Simoa and mass spectrometry, proteins like NfL, Aβ, and p-tau became detectable in blood with high accuracy. However, issues like peripheral interference, comorbidities, and lack of standardization still limit their clinical use. Despite these challenges,

blood biomarkers now represent a major step toward non-invasive diagnosis and monitoring of neurodegenerative diseases. The main types of blood biomarkers in neurodegenerative diseases include amyloid-β (Aβ), phosphorylated tau (p-tau), neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), and ubiquitin C-terminal hydrolase-L1 (UCH-L1). Aβ42/Aβ40 ratio reflects amyloid plaque burden in Alzheimer's disease, while p-tau (especially p-tau181 and p-tau217) indicates tau pathology and disease progression. NfL is a general marker of axonal damage, increasing in many neurodegenerative and neurological conditions. GFAP serves as a marker of astrocytic activation and glial injury, and UCH-L1 reflects neuronal cell body damage. Together, these biomarkers provide valuable information for diagnosis, prognosis, and treatment monitoring in neurodegenerative disorders (Alcolea et al., 2023; Mielke et al., 2018; Janelidze et al., 2020).

Considering the recent advances in blood biomarker research for neurodegenerative diseases, it has become essential to move beyond their diagnostic value and investigate the underlying biological mechanisms responsible for these molecular alterations. In this context, animal and cellular models represent indispensable tools, providing controlled environments to reproduce disease-related processes and to validate the pathophysiological relevance of circulating biomarkers.

Over the last decade, the validation of blood biomarkers and of pathogenetic mechanisms in animal models has significantly advanced our understanding of neurodegenerative disorders and their translational potential. For decades, the understanding of the study of neurodegenerative diseases (NDDs) has been hampered by constraints pertaining to the ability to have access to live, human neurons and the physiological limitations of animal models. Since the invention of human induced pluripotent stem cell (hiPSC) technology, that paradigm has completely transformed, providing a strong resource to study of disease mechanisms in the framework of the patient's own genetic architecture. Through transformation of somatic cells to pluripotent status and its subsequent differentiation into dedicated neuronal or glial subtypes, those early phenotypic patterns of diseases like Alzheimer's (AD), Parkinson's (PD) and amyotrophic lateral sclerosis (ALS), can be recapitulated in vitro (Guo, X., 2025). One of the key strengths of hiPSC-derived models is their ability to uncover early pathological alterations, such as dysregulated autophagy and mitophagy malfunction, well in advance of clinical signs. Recent works stress the importance of hiPSC-based platforms for the discovery of these "invisible" molecular signatures as a bridge between genetic risk factors and observable cellular

insufficiencies (Odonchimed, 2026). Moreover, these models have gained prominence in high-throughput drug screening, through the identification of small molecules that can prevent protein aggregation and/or improve cellular proteostasis in humans (Chang, C. 2020). But research is progressively progressing away from simple two-dimensional (2D) cultures towards more complex three-dimensional (3D) systems, such as cerebral organoids. Although high-resolution molecular analyses dominate in 2D cultures, 3D organoids provide a more accurate representation of the human brain's spatial organization, including layered neurogenesis and complex cell-to-cell interactions (Hou, P, 2022). Moving from 2D to 3D models is a large step towards personalized medicine, as these systems yield more predictive and physiologically relevant information for clinical translation (Centeno, E, 2018). A major challenge in neurodegenerative research is the inherent evolutionary gap between rodent and human central nervous systems. While genetic similarities exist, the intricate functional architecture of the human brain, especially within cognitive circuits, cannot be accurately recapitulated in rodent systems (Dawson T, 2019; Urrestizala-Arenaza N, 2024). Over biological disparities, environmental housing conditions, and experimental design also play a significant role in the lack of reproducibility of results. Moreover, laboratory animals are often housed in artificial environments, devoid of natural light and confined to small cages. The chronic stress induced by these conditions elevates corticosterone levels and alters the expression of inflammation-related genes. Research indicates that even subtle factors, such as bedding material or ambient laboratory noise, can significantly skew behavioural assessments and neurodegeneration outcomes. These uncontrolled environmental variables complicate the translation of findings to humans, who exist in vastly different ecological contexts (Akhtar A, 2015). In light of these limitations, the scientific community is shifting toward alternative models that more closely align with human biology. Anyway, in line with the recent position of the National Institution of Health (NIH) to move away from animal models (https://www.nih.gov/news-events/news-releases/nih-prioritize-human-based-research-technologies) the search for new experimental strategies in human cell-based technologies such as organoids, stem cells, tissue chips and computational models, represent a priority not only from an ethical point of view but also to improve the accuracy in drug development studies to ensure safer therapies and in the study of pathological conditions hard to be translate from animal to humans. Stem cells are basically the body’s master builders. They have this amazing dual power: they can make endless copies of themselves, or they can transform into almost any specific cell the body needs. This is exactly why they are the engine behind regenerative medicine. We’ve moved way beyond just using them as patches for injuries; we’re now using them to build entire lab-grown

environments. This lets us recreate a disease in a dish and see exactly how it works, giving us a real shot at finding a cure (Amin Sharif M, 2025; Fengfeng Li, 2025; Badawy S, 2025). In addition, the ultimate goal here is to connect several of these mini-organs to simulate how the whole body works.

Advances in induced pluripotent stem cell (iPSC) technology have enabled the generation of patient-derived neurons and glial cells, paving the way for personalized and disease-specific studies (Slanzi et al., 2020). Moreover, three-dimensional systems, including organoids and microfluidic “brain-on-a-chip” platforms, have successfully recreated key structural and functional features of the human brain (Pereira et al., 2023). Hu et al. (2010) showed that human iPSCs can differentiate into neuroepithelial structures and electrophysiological active neurons comparable to those derived from embryonic stem cells, while maintaining similar temporal and molecular programs. Although variability in differentiation efficiency was observed among iPSC lines, these findings established the foundation for using iPSC-derived neurons as physiologically relevant models in neurodegenerative disease research.

According to the potential of stem cells, which inspired us to obtain neuronal from them, our project focuses on isolation of stem cells from urine from healthy donors for attempting new novel neuronal models in vitro. USC is easy to establish, cost-effective and patient-specific, and offers an alternative model to investigate rare muscle genetic disorders (Kim EY et al. 2016; Pavathuparambil A et al., 2018). USCs originate from glomerular parietal epithelia although a small fraction also originates from the basal layer of urothelium, from pericytes and from urinary epithelium (Bento G et al. 2020). Moreover, USC display the classical stem cell features, including clonogenicity, cell growth patterns, expansion capacity, stem cell surface marker expression profile and multipotent differentiation (Burdeyron P, et al. 2020).

AIM OF THE PROJECT AND EXPERIMENTAL DESIGN

This project aims to develop a reliable and accessible neuronal cellular model derived from USC of healthy donors to investigate molecular mechanisms involved in neurodegenerative diseases. The project focuses on isolating USCs, inducing their differentiation into neuron-like cells using two independent strategies first small-molecule induction and second NGN2-mediated lentiviral transduction and evaluating the resulting neuronal identity through morphological, molecular, and immunocytochemical analyses. The final goal is to establish a robust in-vitro platform suitable for future applications in disease modelling, biomarker validation, and therapeutic research.

The experimental design of the present thesis project is shown in Figure 1

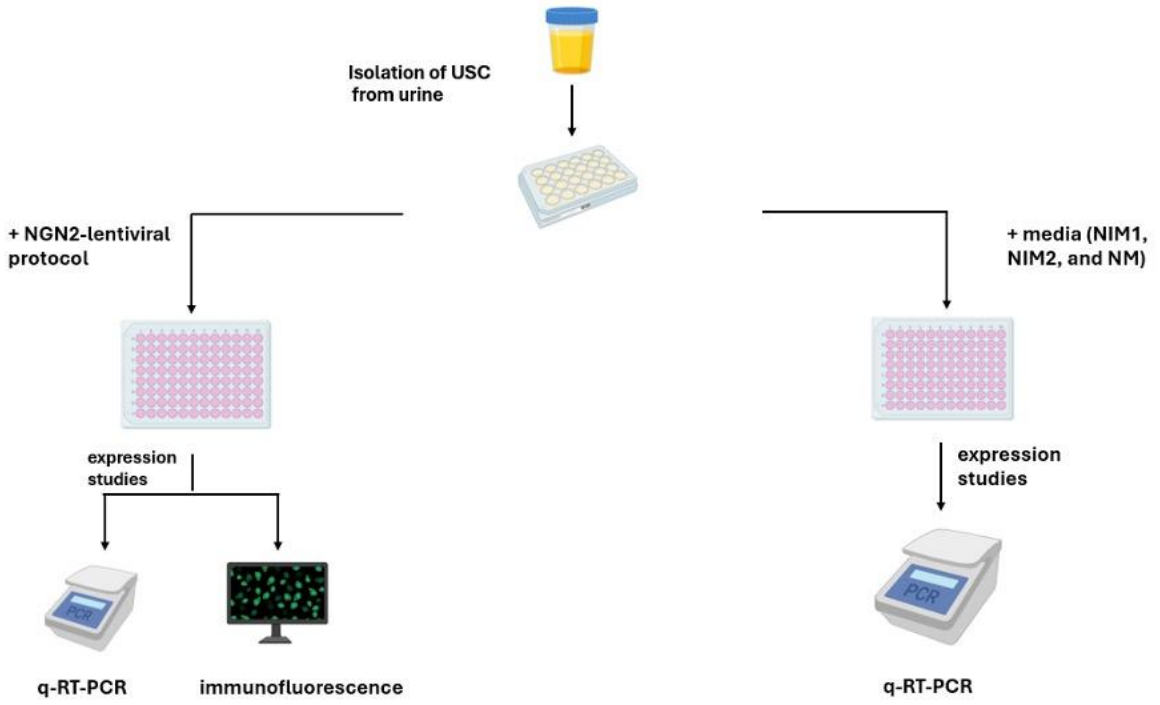

Figure 1. Experimental design of the thesis project

During both protocols, morphological progression was monitored by phase-contrast imaging. Neuronal identity was further confirmed by immunofluorescence for MAP2, DCX, NES, and NGN2, and by qRT-PCR analysis of key neuronal markers including MAP2, TUBB3, TAU,

NCAM, CNTN1, NES, and PAX6. The comparison between protocols enabled the evaluation of differentiation efficiency, maturation level, and cell stability, allowing for the selection of the most suitable strategy for developing a USC-derived neuronal model.

2. METHODS AND MATERIALS

2.1 Cell culture

2.1.1 Urine-derived stem cells isolation and culture

Collection of human urine from healthy volunteers was approved by the local Ethics Committee (Comitato Etico Interaziendale Maggiore della Carità, Novara; authorization CE 190/20). Urine samples were collected from donors and divided into 50 ml centrifuge tubes. 1% penicillin-streptomycin (p/s) was added to the tubes and centrifuged for 10 min at 400 g. Supernatants were then discarded, leaving about 1 ml of sample in each tube. About 10 ml of sterile phosphate-buffered saline (PBS) with 1% p/s was added to the tube to prevent any possible contamination. Then cells were centrifuged again at 200 g for 10 min. Supernatants were discarded, and cells were resuspended with 1 ml of primary medium [DMEM-F12 medium with 1% p/s, 0.1% each supplement from Renal Epithelial Cell Growth (REGM) BulletKit (Lonza, US) and 10% Foetal Bovine Serum (FBS, Gibco)] and transferred into six wells of a gelatin-coated 24-well plate. Every two days, the medium was half-changed. On the fourth day, this medium was substituted by proliferation medium [ratio 1:1 DMEM High Glucose and Renal Epithelial Basal Medium ATCC PCS-400-030, 15% FBS, 1% penicillin-streptomycin, 0.5% L-Ala-L-Glutamine (Euroclone), 0.5% NEAA, 2.5 ng/mL bFGF, 2.5 ng/mL PDGF-BB, 2.5 ng/mL EGF (all Immunotools, GmbH, Gladiolenweg, Germany)]. Proliferation medium was changed on alternate days. Once stem cells have reached 70-80% of confluency, they are passaged in 6-well plates.

2.1.2 Generation of neuron-like cells from urine stem cell by small molecules protocol

USCs were seeded into geltrex-coated plates (1×10^4 cells/cm2) with proliferation medium and culture medium was fully replaced by neuron induction medium 1 (NIM1; Neurobasal medium (Gibco, AUS) supplemented with 1% p/s (Gibco), 1% L-glutamine (Gibco), 2% B27 (Gibco), 1% N2 (Gibco) supplemented with 100 µM cAMP-Na (Sigma), 4 µM Valproic acid (VPA, Calbio-chem), 3 µM CHIR99021 (Selleck chemicals), 1 µM Repsox (Sigma), 10 µM Forskolin (Sigma), 10 µM SP600125 (Sigma), 5 µM GO6983 (Sigma), 10 µM Y-27632 (Sigma),2 µM IBET151 (Sigma), 20 µM Isoxazole9 (ISX9, R&DSystems), 10 µM retinoic acid (RA, Sigma), 5 µM QVD-OPh (MedChemExpress) and 0.2 mM vitamin C (VitC, Sigma)) NIM1 was converted to NIM2 (NIM1 supplemented with 20 ng/ml BDNF, 20 ng/ml GDNF, 20 ng/ml IGF, and 20 ng/ml NT3) by the following day after seeding (Day 0). To reduce cell damage during the media shift, freshly made NIM2 was largely replaced with NIM1

on Day 3. Following Day 11, neuron media (NM; Neurobasal medium, 1% p/s, 1% L-glutamine, 2% B27, 1%N2) supplemented with 20 ng/ml BDNF, 20 ng/ml GDNF, 20 ng/ml IGF, and 20 ng/ml NT3 was used to partially replace the culture medium on alternate days until Day 14.

2.1.3 Generation of neuron like cells from urine-derived stem cell by lentiviral vector transduction

2×10^4 USCs were plated in each well with proliferation medium on geltrex.

USCs have been transduced by lentiviral vectors carrying Neurogenin 2 and rTtA. The transfer vector used for the NEUROG2 lentivirus is hNGN2-P2A-eGFP-T2A-PuroR; i.e., this vector encodes a Tet-O-FUW Advanced transactivator under control of a constitutive TetO promoter and confers resistance to the Ampicillin, 100 µg/Ml. pLV-TetO-hNGN2-eGFP-Puro was a gift from Kristen Brennand (Addgene plasmid # 79823; http://n2t.net/addgene:79823; RRID: Addgene_79823). The transfer vector used for the reverse tetracycline transactivator lentivirus is _FudeltaGW-rtTA; FudeltaGW-rtTA was a gift from Konrad Hochedlinger (Addgene plasmid # 19780; http://n2t.net/addgene:19780; RRID: Addgene_19780). Cells were cultured in DMEM medium (Gibco) supplemented with 1% L-glutamine (Gibco), 1% non-essential amino acids (NEAA; Gibco), 10% foetal bovine serum (FBS; Gibco), 2.5 ng/mL epidermal growth factor (EGF; PeproTech), 2.5 ng/mL fibroblast growth factor (FGF; PeproTech), and 2.5 ng/mL platelet-derived growth factor (PDGF; PeproTech). Doxycycline was added to the culture medium at a final concentration of 4 µg/mL to activate the lentiviral system. Cells were maintained at 37°C with 5% CO_2. The medium was changed every two days.

By day 1, $2x10^4$ mouse astrocytes were plated in complete neuronal induction medium.

On day 2, neurobasal medium was added (Neurobasal medium with 2% (v/v) B-27 supplement, 1% (v/v) L-alanyl-L-glutamine, 1% (v/v) penicillin/streptomycin, NT-3 10 ng/mL, BDNF t 10 ng/mL, doxycycline 4 µg/ mL). 2µL Ara-C was added to the medium for 48h to inhibit astrocytes proliferation. The medium was half changed every two days. From day 9 onwards, the medium was supplemented with 2.5% FBS to support the astrocyte viability.

2.2 RNA extraction

Total RNA extraction from USCs and USC-iNs was performed using TRIzol Reagent (Invitrogen) following standard protocol. Briefly, chloroform was added to the TRIzol-lysed samples (200 µL chloroform per mL of TRIzol used) and the solution mixed by inverting 10-15 times and incubated for 5 minutes at room temperature (RT). Samples were then centrifuged

at 12000 g for 15 minutes at 4°C, and the aqueous phase, containing RNA, was collected and moved to a new tube. The precipitation of the nucleic acid was performed by adding isopropyl alcohol (500 µL per mL of TRIzol used), followed by incubation at RT for 10 minutes and centrifugation at 12000 g for 10 minutes at 4°C. The pellet was washed with 75% ethanol/H2O DEPC and centrifuged at 8000 g for 5 min at 4°C for a total of three times and finally resuspended in diethylpyrocarbonate (DEPC)-treated water using a total volume of 20-50 µL according to the pellet size. RNA samples were then quantified with NanoDrop 2000 (Thermo Scientific) and complementary DNA (cDNAs) obtained using RevertAid H minus first Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol. You add 16 µl water DEPC, and 1 µl for spectrophotometry, and add 4 µl of buffer and 1 µl of enzyme, and the thermocycler for 10 min at 25°C, 15 min at 42°C, and 5 min at 85°C.

2.3 q-RT-PCR

The cDNAs obtained by reverse transcription were used to study the gene expression profile of the populations of interest. Experiments were performed in a 96-well plate containing 10 µl of reaction (5 µL SYBR, 3.2 µL water, 0.4 µL primer) in each well. A CFX96 real-time thermocycler was employed. A three-step cycling real-time PCR was performed (3 minutes 95°C, 5 seconds 95°C and 30 seconds 55°C for 40 cycles) in a volume of 10 µL per well in 96-well optical reaction plates (Bio-Rad, Hercules, California, USA). PCR mix was prepared according to the instructions with 400 nM forward and reverse primers and 1 µL cDNA sample. As control in the experiment analysis, we used neuron progenitor cells kindly gifted by Prof. Mariagrazia Grilli of the Department of Pharmaceutical Sciences.

Table 1. List of primers used for qPCR amplification

Gene	Forward primer (5' to 3')	Revers primer (5' to 3')
NCAM	GGCATCCTCATCGTCATCTT	GAACAGGCCACACTTGTTCA
CNTN1	GATGAAACCATGAGCCCTTC	GGCTGTAAGGTCCATCTCCTT
TUBB3	GGCCAAGGGTCACTACACG	GCAGTCGCAGTTTTCACACTC
NES	CTGCTACCCTTGAGACACCTG	GGGCTCTGATCTCTGCATCTAC
PAX6	TGGGCAGGTATTACGAGACTG	ACTCCCGCTTATACTGGGCTA
TAU	GTCCGTACTCCACCCAAGTC	ATTCTTCAGGTCTGGCATGG
MAP2	CCAATGGATTCCCATACAGG	TCTCCGTTGATCCCATTCTC
GAPDH	AACGTGTCAGTGGTGGACCTG	AGTGGGTGTCGCTGTTGAAGT

2.4 Immunofluorescence (IF) assay

For IF, the day before the start of differentiation, the glass coverslips were sterilised according to the manufacturer's recommendations. Glass coverslips were placed in 24 wells and coated with geltrex 0.1 x 10^5 USC-iN, and USCs were plated on 12mm round coverslips. The following day, cells were fixed with 4% paraformaldehyde (10 minutes at 4°C). Then, the cells were incubated for 1 hour at RT with blocking buffer (5% BSA, 0.1% Triton X-100 in PBS) and then 2 hours with primary antibodies PBS 2% BSA, 0.1% Triton X-100: anti Ngn2 (1:500, PA578556, Life Technologies) anti-microtubule associated protein 2 (MAP2; 1:50, PA517646, Life Tecnologies), anti-nestin (NES; 1:100, MA1110, Life Technologies), anti-doublecortin (DCX; 1:125, 481200, Life Technologies). Then, cells were incubated in the dark for 45 minutes in PBS containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclei detection and secondary antibodies: Goat anti-Mouse IgG Alexa Fluor 546 and Goat anti-Rabbit IgG Alexa Fluor 488 (Invitrogen) diluted 1:500. Images were acquired using DM5500B microscope (Leica). Allow to cure and store in the dark at 4°C.

2.5 Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). A two-tailed unpaired Student's t-test was used to compare the two samples. To compare three or more samples, one-way ANOVA was used, unless otherwise specified. The data are expressed as the mean ± SEM of 'n' independent experiments performed in triplicate, as detailed in the figure legends, and were considered significant at $p<0.05$.

3. RESULTS

3.1 USC-iN differentiated by small molecules

The first attempt at differentiation was carried out by adding different small molecules to the culture medium of USC at different times. As shown Figure 2, neuronal cells presented an elongated shape by day 3 of differentiation.

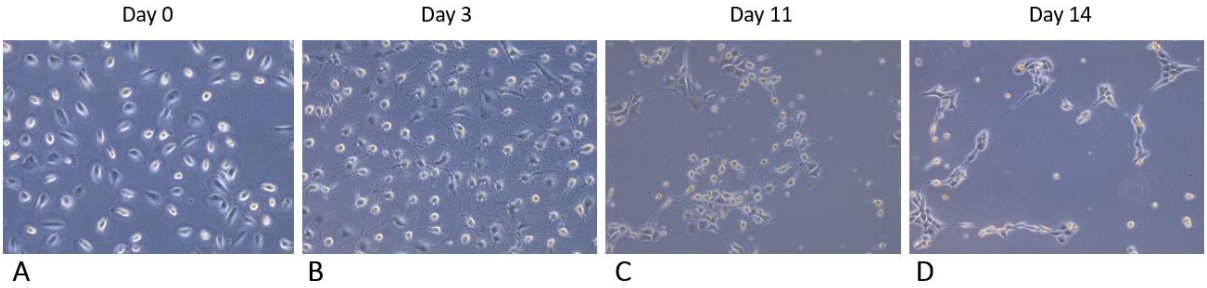

Figure 2. Phase-contrast images of USCs at different stages of differentiation towards neuronal cells (D3, D11, and D14; magnification 20x). A USC's cell after isolation. **B** USC at 3 day after adding NIM1. **C** Neuronal morphologies (as net connection area) after adding NIM2. **D** USC-derived neuron cell like at 14 days.

Although we attempted to measure the neuronal gene expression by q-RT-PCR, the neuronal cells experienced reduced viability after day 11, and they were lost by day 14, limiting further validation by immunofluorescence. Unfortunately, we were not able to explore this aspect extensively, so we modified the protocol accordingly.

3.1.1 Neuronal marker expression evaluation by quantitative RT-PCR:

We used NPCs (neural progenitor cell) as positive control, that were kindly gifted by Prof. Mariagrazia Grilli of the Department of Pharmaceutical Sciences. The MAP2, NCAM, CNTN1, PAX6, NES, TAU, and TUBB3 (Microtubule-associated protein 2, Neuronal cell adhesion molecule, Contantin 1, Paired box gene 6, Nestin, Microtubule-associated tu, βIII-tubulin, respectively) expression were evaluated to confirm neuronal differentiation. All genes were confirmed through qRT-PCR. As reported in Figure 3, non-differentiated cells (USCs) showed a low expression of the genes, while after differentiation, the cells expressed the mRNA for the genes that increase in a time-dependent way.

The RT-qPCR analysis revealed a significant upregulation of MAP2 (Figure 3A). A significant increase was observed in NM D14 compared to the undifferentiated USCs. Notably, the expression levels significantly intensified from the early induction phase (NIM1 D3) to the late maturation stage (NM D14), highlighting the efficiency of the differentiation media. When compared to the positive control group (NPCs), the cells at NIM D10 still showed a lower expression profile, indicating that while neuronal commitment is established early, full maturation is progressively achieved towards the final stages of the protocol. Regarding NCAM expression (Figure 3B), the results demonstrated a robust activation of this neural adhesion molecule during the middle and late stages of differentiation. While no significant difference was observed during the early induction phase, a sharp and highly significant upregulation was detected from day 10 onwards. Furthermore, the comparison between the induction phases showed a steady increase in NCAM levels. Most notably, the expression levels in our differentiated cells at both day 10 and day 14 showed no statistically significant difference when compared to the positive control (NPCs), indicating that our USC-derived cells reached an NCAM expression profile comparable to that of primary neuronal progenitors. Regarding the expression profile of CNTN1 (Figure 3C), although the initial comparison between USCs and the final stage (NM D14) did not reach statistical significance, a significant upregulation was observed when comparing the early induction phase (NIM1 D3) to the mature stage. Interestingly, while the positive control group (NPCs) showed a significantly higher expression compared to the baseline USCs, the difference between our differentiated cells (NM D14) and the NPCs was not statistically significant, indicating that the differentiation protocol effectively induced CNTN1 expression to levels comparable to those found in primary neuronal progenitors. Moreover, PAX6 (Figure 3D) illustrates no significant difference between the undifferentiated USCs and the final stage of the protocol at NM D14. However, a statistically significant increase was observed when comparing the early induction phase at NIM1 D3 to the final maturation stage at NM D14. Analysis of NES (Figure 3E) expression showed no significant difference between undifferentiated USCs and NM D14 at the final maturation stage. During the differentiation process, a statistically significant increase was observed from the early induction phase at NIM1 D3 to the final stage at NM D14. Additionally, the expression levels at NIM2 D10 were found to be comparable to the NPCs, with no statistically significant difference observed between these two groups. TAU (Figure 3F) expression demonstrated a highly significant increase in NM D14 compared to the undifferentiated USCs. Similarly, the positive control group (NPCs) showed a significantly higher expression than the baseline USCs. A robust and highly significant upregulation was

also observed throughout the protocol, specifically when comparing the early induction stage at NIM1 D3 to the final maturation stage at NM D14. Furthermore, no statistically significant difference was found between the intermediate stage at NIM2 D10 and the NPCs, indicating comparable expression levels between these two groups. The expression of TUBB3 (Figure 3G) was significantly higher in NM D14 compared to USCs, while no significant differences were observed between USC and NPCs, NIM1 D3 and NM D14, and NIM2 D10 and NPCs, respectively.

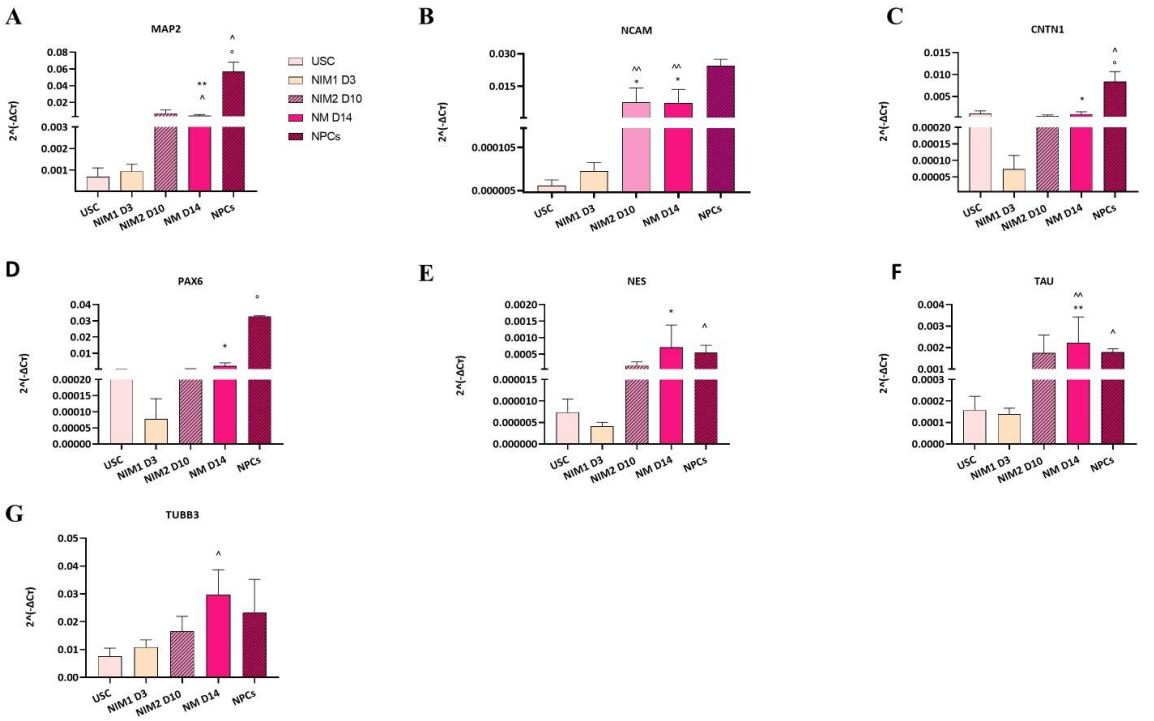

Figure 3. Gene expression analysis during neuronal differentiation of USCs. Relative mRNA expression levels of neuronal markers (A) MAP2, (B) NCAM, (C) CNTN1, (D) PAX6, (E) NES, (F) TAU, and (G) TUBB3 were measured by qRT-PCR. Data are normalised to [GAPDH] and expressed as 2^{-\Delta Ct}. USCs (Urine-derived Stem Cells) represent the undifferentiated baseline, while NPCs serve as a positive control. Data are presented as mean ± SEM (n=3). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ *vs* NIM1 D3. ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$, and °°°° $p < 0.0001$ *vs* NIM2 D10. ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$, and ^^^^ $p < 0.0001$ *vs* USCs. “ns” indicates no statistically significant difference ($p > 0.05$). USC: (Control); D: Day of differentiation.

In particular, qRT-PCR analysis showed a gradual increase in neuronal gene expression during differentiation. Early markers such as NES and PAX6 appeared first, while mature neuronal markers, including MAP2, TUBB3, and TAU, were strongly upregulated at later stages. The rise in NCAM and CNTN1 further confirmed neuronal identity, indicating that urine-derived stem cells successfully differentiated toward a neuronal-like phenotype. Due to the diminished viability of neuron-like cells beyond 11 days of differentiation, a revised protocol utilising lentiviral transduction was implemented.

3.2 USC-iN differentiated by lentivirus infection

In the new protocol, the neuronal differentiation of our cellular model was performed. During the differentiation process, morphological changes were observed under phase-contrast microscopy. As shown in Figure 4, USC progressively acquired neuronal-like morphology through different stages of the new differentiation protocol.

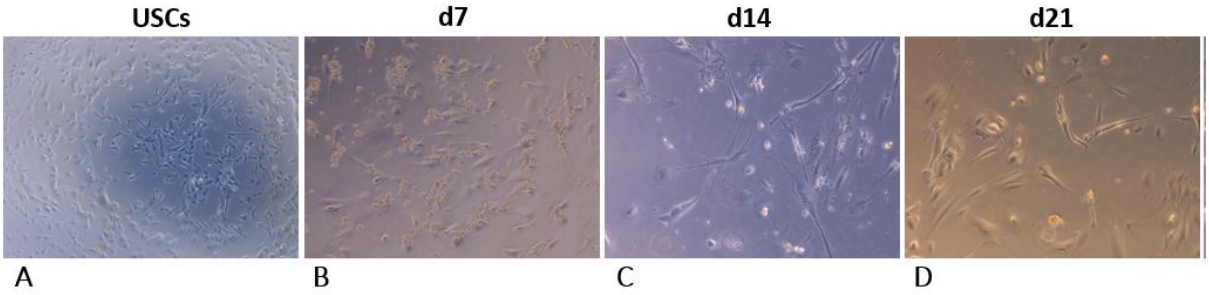

Figure 4: Phase-contrast images at different time of differentiation. (A) Undifferentiated USCs display a compact, fibroblast-like morphology. (B) By day 7, cells begin to extend short neurite-like processes and lose their initial shape. (C) At day 14, cells show elongated bodies and interconnected projections forming early network-like structures. (D) By day 21, the neuronal morphology becomes more defined with multiple branched extensions. Magnification 20×.

USC-iN demonstrated longer vitality, so we were able to proceed with the gene and protein characterisation analyses of the neuronal markers.

3.2.1 Immunofluorescence-Based Assessment of cell differentiation markers

Neuronal markers of differentiation as MAP2, DCX, and NES were first analysed by immunofluorescence assay. As shown in Figure 4, MAP2 (Figure 5A) expression started to

significantly increase at d7 until day 21. In addition. There was a highly significant increase in MAP2 expression from the undifferentiated state (ND) to all subsequent time points. Expression levels rose sharply at Day 7, remained significantly elevated at Day 14, and reached their peak at Day 21 ($P < 0.0001$). Doublecortin (DCX) is a protein generally expressed in stem cells and immature neurons, and this is confirmed by our data as the reduction in the expression of the protein from the USCs is evident (Figure 5B) during the progression of differentiation up to day 21.

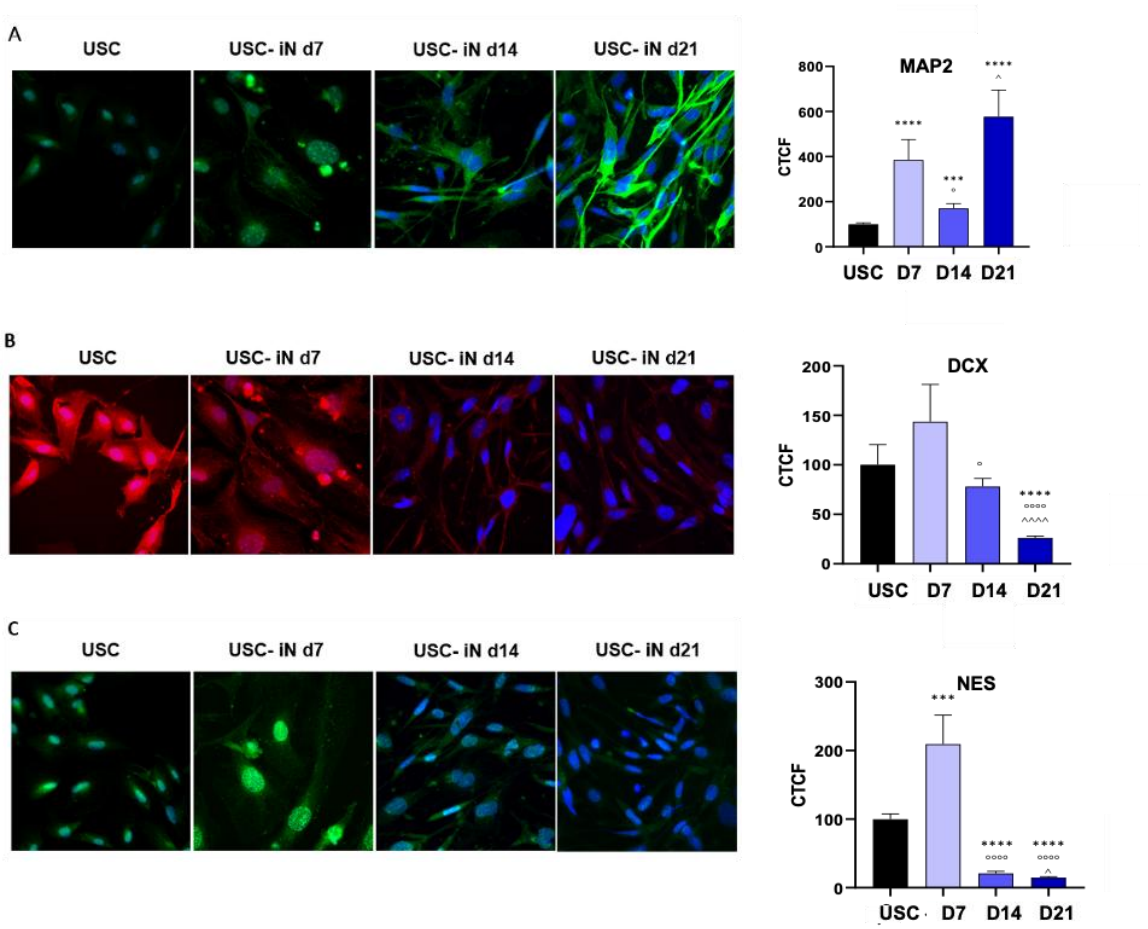

Figure 5, MAP2, DCX, and NES expression in neuronal differentiated cells. Immunofluorescence analysis and quantification of MAP2 (A), NES (B), and DCX (C) at different days of differentiation. Nuclei were stained with DAPI (blue), and secondary antibodies: Goat anti-Mouse IgG Alexa Fluor 546 (Red) and Goat anti-Rabbit IgG Alexa Fluor 488 (green). Magnification 20X. Moreover, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ *vs* USC. ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$, and °°°° $p < 0.0001$ *vs* D7. ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$, and ^^^^ $p < 0.0001$ *vs* D14. "ns" indicates no statistically significant difference ($p > 0.05$). USC: (Control); D: Day of differentiation.

Nestin (NES) (Figure 5C) is a cytoskeletal protein belonging to the intermediate filaments, expressed in progenitor cells, such as stem cells, whose expression tends to decrease as differentiation progresses. Also in this case, the protein expression of nestin decreased as differentiation progressed, as the stem cell, as the days pass, begins to acquire a neuronal phenotype and, therefore, nestin is less and less necessary and therefore less expressed. Moreover, NES and DCX signals were mainly localized in the cytoplasm, indicating the presence of immature and developing neurons.

3.2.2 Gene expression evaluation

We then analysed the gene expression of several neuronal markers, as MAP2 (Figure 6A), PAX6 (figure 6B), NES (Figure 6C), NCAM (Figure 6D), CNTN1 (Figure 6E), TAU (Figure 6F), TUBB3 (Figure 6G) and NGN2 (Figure 6H). Consistently, qRT-PCR results showed a progressive upregulation of **MAP2**, **NCAM**, **PAX6**, **TAU**, **TUBB3**, and **CNTN1** from day 7 to day 21 compared with undifferentiated USCs. These findings demonstrate that urine-derived stem cells successfully differentiated into neuron-like cells under the new induction protocol. Furthermore, MAP2 (Figure 6A) illustrates USC-iN in Days 7, 14, and 21 compared to USC-CTRL, which had significant values. However, day 21 was associated with a clear effect on days 7 and 14. Regarding PAX6 (Figure 6B) expression, a significant increase was observed starting from day 7 compared to the USC control group. This significant difference persisted on day 14, and reached its peak on day 21 ($p=0.0007$), indicating a progressive up-regulation during the later stages of differentiation. In terms of NES (Figure 6C) expression, a significant increase was observed on day 7 compared to the USC group. Although the expression levels remained significantly higher than the control on day 14 and day 21, a critical down-regulation was noted as differentiation progressed. Regarding NCAM (Figure 6D) expression, a significant increase was observed starting from day 7 and day 14 compared to the USC control group. However, the most prominent up-regulation occurred on day 21, showing an extremely significant difference compared to the USC group.

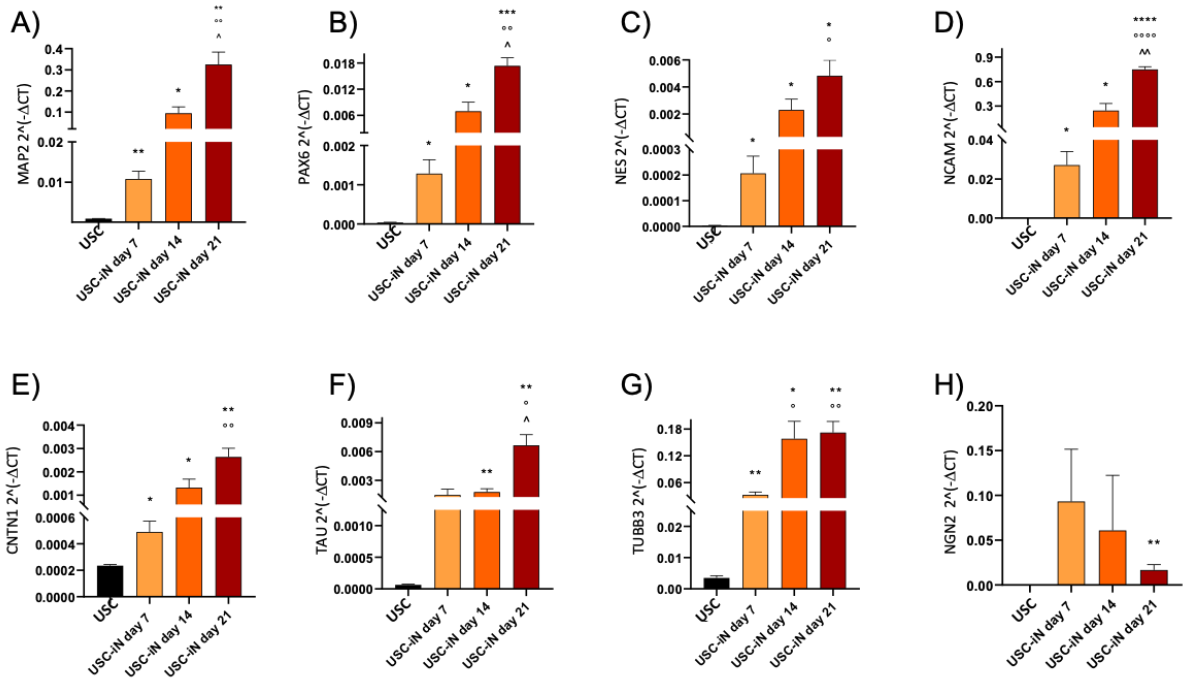

Figure 6. Quantitative analysis of neural marker expression during differentiation. (A–H) mRNA expression levels of MAP2, PAX6, NES, NCAM, CNTN1, TAU, TUBB3, and NGN2 were measured at four time points (USC, D7, D14, and D21) using qPCR (2^{-\Delta\Delta Ct} method). Data are presented as mean ± SEM. Statistical significance was analyzed by one-way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ *vs* USC. ° $p < 0.05$, °° $p < 0.01$ *vs* D7. ^ $p < 0.05$, ^^ $p < 0.01$ *vs* D14. USC: (Control); D: Day of differentiation.

CNTN1 (Figure 6E) expression revealed a significant up-regulation starting from day 7 and day 14 compared to the USC control group. This increase became more pronounced by day 21, showing a highly significant difference relative to the USC group. These data suggest a steady accumulation of CNTN1 as the cells progress toward a neuronal phenotype. Then, examining the neuronal marker TUBB3 (Figure 6F) revealed a robust and early response during the differentiation process. A marked up-regulation was initiated as early as day 7, which was further intensified by day 14 and day 21, showing a highly significant difference compared to the control group. In contrast to the earlier markers, the expression profile of NGN2 (Figure 6H) exhibited a high degree of stability throughout most of the differentiation period. No significant deviations were observed on day 7 or day 14 when compared to the USC control group. However, a significant emergence in expression was finally detected by day 21.

4. DISCUSSION

Neurodegenerative diseases (NDDs) represent a diverse class of disorders characterised by the gradual loss of neuronal structure and function, including disruptions in axons, dendrites, myelin integrity, and intracellular signalling pathways (Heemels, 2016). To date, a great number of dementia patients are not responsive to the currently used treatments (Agnello & Ciaccio, 2022). Understanding the molecular events underlying these alterations requires reliable and accessible neuronal models, which motivated the development of the USC-derived neuronal model to make it easier to work on laboratory models and monitor their vulnerability. A significant portion of the discussion should be dedicated to the rationale for selecting USCs over other somatic sources such as skin fibroblasts or peripheral blood mononuclear cells (PBMCs). USCs are uniquely positioned at the intersection of accessibility and biological utility. They can be obtained through a simple, painless, and repeatable procedure, which significantly increases patient compliance, particularly in elderly populations suffering from cognitive decline (Gamal A. Atia, 2025). USCs are easy-to-access stem cells since urine collection does not require invasive procedures, the cell isolation protocol is straight-forward and stem cell colonies appear in 2-4 weeks. Interestingly, we have previously demonstrated that the isolation procedure can be performed from fresh urine as well as from urine stored at 4°C for 24 hours, without increasing contamination risk or affecting the efficiency of the process and the quality of USCs (Talmon et al, Cell Calcium 2022). Moreover, we have the source of non-invasive and patient-specific, and the possibility to collect specimens without moving donors and thus to increase the compliance of patients to have access to an easier model in vitro. Furthermore, the possibility to transdifferentiate USCs into neuronal-like cells reduces the *in vitro* manipulations necessary to establish the cellular model that could represent a limit for genetic diseases studies. This work creates a dependable neuronal model derived from USC, providing an accessible platform for examining the molecular pathways implicated in neurodegenerative disorders.

In the first protocol, we observed the neuronal viability, but they suffered after day 10, so the immunofluorescence failed, and the cells could not be maintained any further. Anyway, the cells demonstrated the expression of MAP2, a neuronal cytoskeletal protein found in dendrites, and it is an indicator of mature neuronal development. Whenever MAP2 increase significantly, the cells are distinguished as mature neuronal, so the evidence suggests that this distinction occurs on day 10 after we added NIM2 solution, and the expression reached its maximum at NM D14, showing a statistically significant upregulation, probably as a consequence of the

coordinated action of the small-molecule inducers included in this protocol. In particular, VPA, through its HDAC-inhibitory activity, promotes chromatin relaxation and facilitates the activation of neuronal gene programs; retinoic acid contributes to neuronal maturation and dendritic elaboration, while the cAMP–forskolin axis supports neurite extension and cytoskeletal organisation. Moreover, the neurotrophic factors supplemented during the NIM2 and NM phases (BDNF, GDNF, IGF and NT3) are known to sustain neuronal survival and enhance dendritic complexity. Altogether, these components likely converged in promoting the significant MAP2 upregulation observed at NIM2 D10 and NM D14, before the subsequent decline in cell viability prevented further maturation analyses. Furthermore, NCAM, helps in the formation, connections in neuronal cells and is important in synaptic plasticity. If it increases, it means the cells are in the building the net connections phase. Moreover BDNF, GDNF, NT3 and IGF are well-known to sustain neuronal survival, enhance neurite extension and strengthen cell–cell adhesion, therefore all of which directly contribute to the upregulation of adhesion molecules such as NCAM. Additionally, the activation of the cAMP–forskolin signalling pathway supports neurite outgrowth and cytoskeletal organisation, while retinoic acid and ISX9 promote neuronal differentiation and synaptic maturation. Interestingly, previous studies have shown that USCs express neural-cell adhesion molecules such as NCAM even in the undifferentiated state. For instance, Falzarano et al. (2021) demonstrated that USCs express hundreds of genes related to neuromuscular diseases, indicating a predisposition for neural adhesion and network-related functions. This observation supports our finding of relatively high basal CNTN1 expression in USCs, suggesting that the intrinsic adhesive and neural-progenitor-like properties of USCs may underlie the observed baseline level of CNTN1. Instead, PAX6 has a role in neural progenitor cells, and it shows that the cells go to neuronal pathways. We observed that it increases on day 10 and significantly on day 14 compared to day 10, and we can also see the high levels of Tau, which played a role in axon and maturation on day 14. Maybe we have cells at different levels of differentiation in plates, so the immature cells can affect the final analysis. Furthermore, NES, which is an intermediate filament protein found in neural stem cells, shows the early stage of neurogenesis. We found that high expression occurred on day 14. In addition, TUBB3, which is a strong neuronal marker, is indicative of neuronal cytoskeleton formation, which increases during early to mid-differentiation. Our findings showed that the TUBB3 boost occurred on day 14.

Fortunately, in the second protocol, it became evident that all these genes were also expressed on days 7, 14, and 21; however, the high level of expression was observed on day 21 with greater stability. In addition, we quantified NGN2, which is inserted into cells by a lentiviral vector, and it is an important factor for neuronal transcription and neurogenesis. The immunofluorescence analysis provided further confirmation of the molecular findings obtained by qRT-PCR, revealing a coherent progression of neuronal identity across the differentiation stages. In the first protocol, immunofluorescence could not be fully carried out due to the marked decline in cell viability after day 10, preventing a reliable assessment of protein-level expression. By contrast, in the second protocol, the sustained viability and morphological stability of the cells enabled a clear temporal resolution of neuronal marker expression. MAP2 progressively increased from day 7 to day 21, reflecting dendritic maturation, whereas the early markers DCX and NES displayed strong signals at day 7 followed by a gradual reduction at later stages, consistent with the transition from immature to mature neuronal phenotypes. This pattern mirrors the transcriptional profile observed in qRT-PCR, in which MAP2, TUBB3, TAU, NCAM and CNTN1 were all upregulated at later stages, while progenitor-associated markers showed a relative decline. The findings indicate the early markers, Nestin (NES) and Doublecortin (DCX), are at their maximal levels at day 7 and drastically reduced by day 21. This decrease is critical to the argument as it indicates that the USCs are not receiving only a neuron-like phenotype but are successfully leaving the progenitor state. NES is an intermediate filament protein which is predominantly expressed by proliferating neural stem cells, while DCX is a microtubule-associated protein that is critical for neuronal migration in the developing brain. Their reduction indicates a consistent biological transition toward terminal differentiation, a process that was greatly more stable in the NGN2-mediated protocol than in the small-molecule attempt. The concordance between immunofluorescence and gene-expression data strongly supports the conclusion that USC-derived cells underwent a structured and stage-dependent neuronal differentiation process, with the NGN2-based protocol providing a far more efficient and stable conversion compared to the small-molecule induction approach. The observed expression of neuronal adhesion genes such as CNTN1 and NCAM in USC-derived neurons establishes a framework for validating blood- or CSF-based biomarkers. In clinical practice, proteins like neurofilament light chain (NfL), p-tau, and NCAM are used to monitor axonal damage and disease progression. By correlating the levels of these markers in the in vitro USC-derived neurons with the clinical status of the donor, researchers can gain a more nuanced understanding of how circulating biomarkers reflect cellular-level dysfunction (Madhurima Chatterjee, 2018).

A fundamental theme of concern, the difference in survival and maturation between the small molecule protocol and the NGN2 transduction strategy is one worthy of detailed elucidation. At first, on day three, USCs applied a mixture of small molecules (VPA, CHIR99021, Repsox, etc.) presented with elongated, neuron-like morphologies, yet cellular viability rapidly decreased after day 11, precluding long-term validation through immunofluorescence. This phenomenon is not unique; it points to a more general problem in pharmacologic reprogramming. While small molecules exert their effects by regulating signaling pathways and inhibiting epigenetic gatekeeping, such as histone deacetylases (HDACs), reaching the perfect stoichiometric equilibrium necessary for terminal differentiation has long been a challenge. The extensive metabolic and proteotoxic stress caused by prolonged exposure to high-concentration pharmacological media often activates apoptotic pathways prior to the emergence of functional maturity. Moreover, chemical induction, according to literature, is often accompanied by partial reprogramming, whereby cells express a subset of neuronal proteins instead of acquiring the electrophysiological competence (e.g. the ability to fire repetitive action potentials) associated with bona fide neurons. In contrast, the NGN2-mediated protocol showed long-term viability for over 21 days, providing definitive evidence of the presence of intricate, interconnected network structures. This approach works best because Neurogenin-2 (NGN2) is a biological "pioneer factor." In contrast to other types of transcription factors, which can only interact with portions of the genome that already have that information (open chromatin), pioneer factors are able to connect to target regions within condensed closed chromatin. The mechanistic transition induced by NGN2 involves multilayered chromatin remodeling at dynamic enhancer-gene interaction sites. NGN2 is reported to rewire the overall 3D genome architecture, establishing enhancer-promoter interactions characteristic of different cell types, that are critical for the neuronal program as demonstrated by the above-mentioned research. Of particular relevance, NGN2 has been shown to recruit the transcriptional co-factor Yy1, an adaptor protein that regulates chromatin opening, and thus may activate neurogenic genes. This stability of the NGN2 protocol in the experimental results resonates with the observation that NGN2-induced neurons (iNs) quickly skip the progenitor phase and convert the somatic identity directly into a neuronal one without the stochasticity of small-molecule media (Allwyn Pereira, 2022).

In conclusion, the NGN2-based protocol ensured sustained survival of differentiated neuronal-like cells and enabled a coherent temporal progression of gene and protein expression, with early neurogenic markers declining and mature neuronal markers consistently increasing up to day 21. The concordance between qRT-PCR and immunofluorescence strongly supports the notion that NGN2 overexpression provides a more robust and reliable strategy for driving USC-derived cells toward a stable neuronal identity. The use of USCs as an accessible and patient-specific cell source has been progressively validated over the past decade, with multiple studies demonstrating their multipotency, proliferation capacity, and suitability for modelling human diseases. The present thesis aligns with this body of literature by confirming that USCs can be redirected toward a neuronal fate and expands these findings by providing a temporal transcriptional characterisation of neurogenic commitment under two distinct differentiation paradigms.

5. Bibliography

Abdul Manaph, N.P., Al-Hawwas, M., Bobrovskaya, L., Coates, P.T. and Zhou, X.F. (2018) 'Urine-derived cells for human cell therapy', *Stem Cell Research & Therapy*, 9(1), p. 189.

Agnello, L. and Ciaccio, M. (2022) 'Neurodegenerative diseases: from molecular basis to therapy', *International Journal of Molecular Sciences*, 23(21), p. 12854.

Akhtar, A. (2015) 'The flaws and human harms of animal experimentation', *Cambridge Quarterly of Healthcare Ethics*, 24(4), pp. 407–419.

Alcolea, D., Beeri, M.S., Rojas, J.C., Gardner, R.C. and Lleó, A. (2023) 'Blood biomarkers in neurodegenerative diseases: implications for the clinical neurologist', *Neurology*, 101(4), pp. 172–180.

Atia, G.A., Abdal Dayem, A., Taher, E.S., Alghonemy, W.Y., Cho, S.G., Aldarmahi, A.A., Haque, M.A. et al. (2025) 'Urine-derived stem cells: a sustainable resource for advancing personalized medicine and dental regeneration', *Frontiers in Bioengineering and Biotechnology*, 13, p. 1571066.

Bento, T.P.F., Genebra, C.V.S., Maciel, N.M., Cornelio, G.P., Simeão, S.F.A.P. and Vitta, A. (2020) 'Low back pain and some associated factors: is there any difference between genders?', *Brazilian Journal of Physical Therapy*, 24(1), pp. 79–87.

Bharadwaj, S. et al. (2013) 'Multipotential differentiation of human urine-derived stem cells: potential for therapeutic applications in urology', *Stem Cells*, 31(9), pp. 1840–1856.

Burdeyron, P., Giraud, S., Hauet, T. and Steichen, C. (2020) 'Urine-derived stem/progenitor cells: a focus on their characterization and potential', *World Journal of Stem Cells*, 12(10), pp. 1080–1096.

Centeno, E.G.Z., Cimarosti, H. and Bithell, A. (2018) '2D versus 3D human induced pluripotent stem cell-derived cultures for neurodegenerative disease modelling', *Molecular Neurodegeneration*, 13(1), p. 27.

Chang, C.Y. et al. (2020) 'Induced pluripotent stem cell (iPSC)-based neurodegenerative disease models for phenotype recapitulation and drug screening', *Molecules*, 25(9), p. 2000.

Chatterjee, M., Schild, D. and Teunissen, C.E. (2019) 'Contactins in the central nervous system: role in health and disease', *Neural Regeneration Research*, 14(2), pp. 206–216. Available at: https://doi.org/10.4103/1673-5374.244776.

Dash, U.C. et al. (2025) 'Oxidative stress and inflammation in the pathogenesis of neurological disorders: mechanisms and implications', *Acta Pharmaceutica Sinica B*, 15(1), pp. 15–34.

Dawson, T.M., Golde, T.E. and Lagier-Tourenne, C. (2018) 'Animal models of neurodegenerative diseases', *Nature Neuroscience*, 21(10), pp. 1370–1379.

Dematteis, G. et al. (2025) ‘ATM knock out alters calcium signalling and augments contraction in skeletal muscle cells differentiated from human urine-derived stem cells’, *Cell Death Discovery*, 11(1), p. 177.

Hou, P.S. and Kuo, H.C. (2022) ‘Central nervous system organoids for modeling neurodegenerative diseases’, *IUBMB Life*, 74(9), pp. 813–827.

Firdaus, R. et al. (2024) ‘Life cycle assessment of rice straw for energy valorization: a comprehensive review of methodological trends and future outlooks’, *WIREs Energy and Environment*, 13(4), e520.

Forrest, S.L. and Kovacs, G.G. (2025) ‘Current concepts and molecular pathology of neurodegenerative diseases’, *Pathology*, 57(2), pp. 178–190.

Frega, M. et al. (2017) ‘Rapid neuronal differentiation of induced pluripotent stem cells for measuring network activity on micro-electrode arrays’, *Journal of Visualized Experiments*, (119), e54900.

Fu, H., Hardy, J. and Duff, K.E. (2018) ‘Selective vulnerability in neurodegenerative diseases’, *Nature Neuroscience*, 21(10), pp. 1350–1358.

Gonzalez-Rodriguez, P., Zampese, E. and Surmeier, D.J. (2020) ‘Selective neuronal vulnerability in Parkinson's disease’, *Progress in Brain Research*, 252, pp. 61–89.

Gorelick, P.B. et al. (2011) ‘Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the American Heart Association/American Stroke Association’, *Stroke*, 42(9), pp. 2672–2713.

Guo, X., Wang, X., Wang, J., Ma, M. and Ren, Q. (2025) ‘Current development of iPSC-based modeling in neurodegenerative diseases’, *International Journal of Molecular Sciences*, 26(8), p. 3774.

Heemels, M.-T. (2016) ‘Neurodegenerative diseases’, *Nature*, 539(7628), pp. 179–179.

Ho, S.-M. et al. (2016) ‘Rapid Ngn2-induction of excitatory neurons from hiPSC-derived neural progenitor cells’, *Methods*, 101, pp. 113–124.

Janelidze, S. et al. (2020) ‘Plasma P-tau181 in Alzheimer’s disease: relationship to other biomarkers, differential diagnosis, neuropathology and longitudinal progression to Alzheimer’s dementia’, *Nature Medicine*, 26(3), pp. 379–386.

Kampmann, M. (2024) ‘Molecular and cellular mechanisms of selective vulnerability in neurodegenerative diseases’, *Nature Reviews Neuroscience*, 25(5), pp. 351–371.

Kim, E.Y. et al. (2016) ‘Compound EGFR mutation is frequently detected with co-mutations of actionable genes and associated with poor clinical outcome in lung adenocarcinoma’, *Cancer Biology & Therapy*, 17(3), pp. 237–245.

Li, F. and Fu, C. (2025) ‘Stem cell revolution: bridging the gap between research and clinical application’, *Stem Cell Research & Therapy*, 16(1), p. 661.

Menéndez-González, M. (2023) ‘Toward a new nosology of neurodegenerative diseases’, *Alzheimer's & Dementia*, 19(10), pp. 4720–4726.

Mielke, M.M. et al. (2018) ‘Plasma phospho-tau181 increases with Alzheimer's disease clinical severity and is associated with tau-PET and amyloid-PET’, *Alzheimer's & Dementia*, 14(8), pp. 989–997.

Odonchimed, S., Imamura, K. and Inoue, H. (2026) ‘Neurodegenerative disease and autophagy in iPSC-based models’, *Neuroscience Research*, 222, p. 104991.

Pereira, A. et al. (2024) ‘Direct neuronal reprogramming of mouse astrocytes is associated with multiscale epigenome remodeling and requires Yy1’, *Nature Neuroscience*, 27, pp. 1260–1273.

Pihlstrøm, L., Wiethoff, S. and Houlden, H. (2018) ‘Genetics of neurodegenerative diseases: an overview’, in Kovacs, G.G. and Alafuzoff, I. (eds.) *Handbook of Clinical Neurology*, Vol. 145. Elsevier, pp. 309–323. *(Book chapter)*

Sharifi, M.A., Yasin, N.F., Kamarul, T. and Sharifi, A.M. (2025) ‘The regenerative journey: exploring stem cell roles from injury detection to tissue repair’, *Stem Cell Research & Therapy*, 16(1), p. 541.

Slanzi, A., Iannoto, G., Rossi, B., Zenaro, E. and Constantin, G. (2020) ‘In vitro models of neurodegenerative diseases’, *Frontiers in Cell and Developmental Biology*, 8, p. 328.

Subramaniam, S. (2019) ‘Selective neuronal death in neurodegenerative diseases: the ongoing mystery’, *The Yale Journal of Biology and Medicine*, 92(4), pp. 695–705.

Surmeier, D.J., Obeso, J.A. and Halliday, G.M. (2017) ‘Selective neuronal vulnerability in Parkinson disease’, *Nature Reviews Neuroscience*, 18(2), pp. 101–113.

Teleanu, D.M., Niculescu, A.G., Lungu, I.I., Radu, C.I., Vladâcenco, O., Roza, E., Costăchescu, B., Grumezescu, A.M. and Teleanu, R.I. (2022) ‘An overview of oxidative stress, neuroinflammation, and neurodegenerative diseases’, *International Journal of Molecular Sciences*, 23(11), p. 5938.

Urrestizala-Arenaza, N., Cerchio, S., Cavaliere, F. and Magliaro, C. (2024) ‘Limitations of human brain organoids to study neurodegenerative diseases: a manual to survive’, *Frontiers in Cellular Neuroscience*, 18, p. 1419526.

Young, P.N.E. et al. (2020) ‘Imaging biomarkers in neurodegeneration: current and future practices’, *Alzheimer’s Research & Therapy*, 12(1), p. 49.

Zhang, Y. et al. (2013) ‘Rapid single-step induction of functional neurons from human pluripotent stem cells’, *Neuron*, 78(5), pp. 785–798.

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