Serotonin analysis in human platelets for the early diagnosis of Parkinson's disease

Final Grade Project

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RESUMEN

Enfermedades neurodegenerativas con una prevalencia significativa en la población, como la Enfermedad de Parkinson (EP), carecen de un método eficaz de diagnóstico precoz. Aún hoy, el diagnóstico de la EP es fundamentalmente clínico y los primeros síntomas aparecen cuando ya ha ocurrido una gran destrucción neuronal.

La dopamina (DA) es el principal neurotransmisor involucrado en la patogénesis de la EP y su pérdida, la responsable mayoritaria de los síntomas. Tanto la DA como su metabolito principal (DOPAC), son extremadamente citotóxicos, así que se almacenan en vesículas secretoras dentro de las neuronas dopaminérgicas. La DA se acumula en gránulos de secreción principalmente gracias a la acción de tres transportadores vesiculares: la bomba de protones vesicular que es una ATPasa (V-ATPasa), el transportador de monoaminas vesicular (VMAT) y el transportador vesicular de nucleótidos (VNUT). Estos tres sistemas son comunes a otras vesículas de secreción de células periféricas como las células cromafines, los mastocitos y las plaquetas, que almacenan catecolaminas, histamina y serotonina (5-HT) respectivamente.

En este trabajo, se han definido y optimizado los protocolos de aislamiento y mantenimiento funcional de cultivos de plaquetas aisladas. Se validaron también los métodos óptimos para determinar tanto el contenido basal como la capacidad de captación de 5-HT por parte las plaquetas. Además, se compararon los valores de concentración de 5-HT en muestras de pacientes con EP y en muestras de voluntarios sanos, dando los primeros pasos en lo que podría ser un método eficaz de diagnóstico preclínico de la Enfermedad de Parkinson.

Palabras clave: Parkinson, dopamina, gránulos de secreción, plaquetas, serotonina.

ABSTRACT

Parkinson's Disease (PD) is a neurodegenerative disease that does not have an effective method for its early diagnosis. Currently, the only way to identify PD is through its clinical manifestations, appearing once a large degree of neuronal destruction has already occurred.

Dopamine (DA) is the main neurotransmitter involved in the pathogenesis of PD and its loss is the main cause of symptoms. Both DA and its main metabolite (DOPAC), are extremely cytotoxic, so they must be stored in secretory vesicles inside dopaminergic neurons. DA is accumulated in secretory vesicles by means of three main vesicular transporters: the proton vesicular carrier that is an ATPase (V-ATPase), the vesicular monoamine transporter (VMAT) and the vesicular nucleotide transporter (VNUT). These systems are also common to other secretory vesicles from peripheral cells, such as chromaffin, mastocytes and platelets, which pack catecholamines, histamine and serotonin, respectively.

Through this project we have optimized the methods for isolation and functional analyses of human platelets in culture. We have also analysed the content and uptake of serotonin concentrations in human platelets from PD patients and supposedly healthy volunteers. The proposed methods could be an effective technique of preclinical diagnosis of Parkinson's disease.

Key words: Parkinson, dopamine, secretory vesicles, platelets, serotonin.

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INTRODUCTION

1. PARKINSON'S DISEASE

1.1. GENERAL CONCERNS

After Alzheimer's, Parkinson's disease (PD) is the most common neurodegenerative disorder. It is classically characterized by a degeneration of *sustantia nigra pars compacta* (SNpc) and the presence of Lewy bodies, with dopamine (DA) deficiency located on basal ganglia, which lead to a defect in motor control. The incidence of this condition is between 10-18 new cases per 100,000 people/year, being interesting to highlight the relation among men-women what is approximately 3:2, thereby gender may have an influence on the development of PD. Also, ethnicity needs to be considered inasmuch as it influences on the prevalence. It is higher in Europe (66-1,500 per 100,000), North America (111-329 per 100,000) and South America (31-470 per 100,000) (Kalia & Lang, 2015). Despite that, the major risk for developing PD is the age. It is estimated that the prevalence of PD is around 1% in population above 60 years old, and around 4% in the one above 85 years old. Thus, considering the increase of live expectancy, it is predictable that this prevalence will increase 50% for 2030 (Driver et al., 2009; Kalia & Lang, 2015).

1.2. CLINICAL APPROACH

PD encompasses classical, but heterogeneous, motor symptoms (first defined by James Parkinson in the 19th century and later refined by Jean-Martin Charcot), such as bradykinesia, muscular stiffness, rest tremor and postural and gait deterioration. In addition, to some non-motor events, consisting of: olfactive dysfunction, cognitive impairment, psychiatric symptoms, sleeps disorders, autonomic dysfunction, pain and fatigue. Besides that, about 83% of at least 20-years-lasting PD patients suffer from dementia (Hely et al., 2005; Kalia & Lang, 2015).

These non-motor events are typically taking place in PD before the beginning of classical motor symptoms, being known as premotor phase, which can have around 12-14 years of latency and might be vitally important for early diagnosis and treatment of PD (Kalia & Lang, 2015).

1.3. DIAGNOSIS AND TREATMENT

Despite Gold Standard for diagnosis of PD is the neurological evaluation, currently there are not standardized criteria. The use of imaging techniques, such as positron-emission tomography (PET) or single-photon emission computed tomography (SPECT), might show altered results only when there is a substantial loss of dopaminergic neurons, being both ineffective for early diagnosis. Probably the most specific neuroimaging technique is the 123I-ioflupane SPECT (DaTSCAN), because it allows to make a differential diagnosis between PD and non-degenerative tremors. Withal, despite significant evidence for the utility of neuroimaging in assessing parkinsonian patients, none of the neuroimaging techniques are specifically recommended for routine use in clinical practice (Pagano et al., 2016).

Genetic tests may help for those who have relatives affected by a known-monogenic type of PD. However, despite as most of them have an incomplete penetrance, a positive result on an asymptomatic individual, does not provide a determinate diagnosis (Kalia & Lang, 2015).

Therefore, nowadays the diagnosis of PD is mainly clinical, based on the appearance of classical motor symptoms: bradykinesia, rigidity and rest tremor. The diagnosis is confirmed when there is a favourable response to an empiric treatment with L-DOPA. It must be considered that these clinical symptoms would only be showed when there is a 50-60% loss of dopaminergic neurons and a 70-80% of dopamine depletion. Recent findings have proved that a moderated loss of nigrostriatal neurons also happens in early phases of PD, being a proportion of them, potentially rescued (Kalia & Lang, 2015).

According to all these facts, establishing a method for an early diagnosis of PD will be the key for monitoring the course of the condition and to set a preventive treatment for slowing down its progression.

As regards to the treatment, drugs that increase neuronal DA concentration or stimulate their receptors are still the bases for dealing with motor symptoms. These drugs currently include levodopa, dopaminergic agonists, type B monoaminoxidase inhibitors (such as selegiline and rasagiline) and amantadine.

1.4. THEORIES OF PARKINSON'S DESEASE ORIGIN

1.4.1. Environmental Exposures

Some substances and life habits significantly alter the probability of PD to be developed. Therefore, 11 main environmental risk factors have been identified so far and exposed below:

Increased risk (OR>1)	***Decreased risk (OR<1)***
Pesticide exposure	*Tobacco smoking*
Prior head injury	*Coffee drinking*
Rural living	*Non-steroidal anti-inflammatories*
β-blockers use	*Calcium channel blockers use*
Agricultural occupation	*Alcohol consumption*
Well-water drinking	*Frequent aerobic exercise*

However, some other considerations might be taken into account. For instance: welding and manganese exposure are not related to a hazard raise. As a suspicion, antipsychotics (phenothiazines, benzamides, haloperidol or risperidone) and solvents (trichloroethylene) enhance risk of PD, but confirmation is yet being required (Kalia & Lang, 2015).

1.4.2. Genetic factors

There is a family background in around 15% of the total PD's patients. Thus, those with family history of Parkinson or tremor have more risk to develop PD, showing that PD is related to genetic factors, among others. At least 23 loci and 19 genes that cause the condition are currently known, 10 of which are autosomal dominant, while 9 are recessive (Nalls et al., 2018).

The first gene associated with a familiar or hereditary way of PD, was SNCA (its mutations are related to autosomal dominant parkinsonism), which codifies α-synuclein (Polymeropoulos et al., 1997). This presynaptic protein turns to be the major component of Lewy bodies and neurites. SNCA's mutations bestow upon α-synuclein neurotoxic functions, easing its aggregation. As a result of this accumulation, soluble monomers and

insoluble fibrils of this protein are formed. Between them, soluble and oligomeric forms cause the worse toxic harm (Soto, 2012; Kalia & Lang, 2015).

Another causing-disease gene is LRRK2, being the most common cause of genetic autosomal PD. LRRK2 is a multidomain protein, whose mutations lead to an altered GTPase and kinase activity, damaging various signalling pathways responsible of vesicular traffic, cytoskeleton and lysosomal function and protein synthesis (Henry et al., 2015). The interplay between environmental and genetic risk factors is yet under investigation.

1.4.3. Neurotoxicity theory

Dopamine (DA) is the main neurotransmitter involved in the pathogenesis of PD and its loss is the responsible for most of its symptoms. DA is a catecholamine family member, synthesized in a restricted set of cell types, mainly neurons from the ventral tegmental area of the midbrain, *sustantia nigra pars compacta* (SNpc) and *hypothalamus arcuate nucleus*, along with the medulla of the adrenal glands (Seeman, 2009).

DA is the result of a two-step-process consisting of the hydroxylation of L-tyrosine to produce L-DOPA through tyrosine-hydroxylase (TH), followed by its decarboxylation via aromatic-L-amino acid decarboxylase (LAAD) (Broadley, 2010). In neurons, this synthesis takes places at the axon terminals, where DA is packaged in vesicles until its release as an answer of presynaptic stimuli (Zhen Qi, 2008).

Dopamine is broken down into inactive metabolites by a set of enzymes -MAO and COMT -, leading to two main different breakdown pathways (**Fig. 01.**) whose principal end-product is homovanillic acid (HVA), which has no known biological activity (Eisenhofer et al., 2004). Cytosolic DOPAC can be oxidized by hydrogen peroxide, bringing about the formation of toxic metabolites, which destroy dopamine neurons in the *substantia nigra*. This neuronal death contributes to the failure of levodopa treatment. MAO-B inhibitors such as selegiline or rasagiline seem to prevent this neurotoxicity (Eisenhofer et al., 2004).

We can deduce that both DA and its main metabolite -DOPAC- are extremely cytotoxic when they are located free at the cytosol. Because of that, DA must be stored in secretory vesicles inside dopaminergic neurons, preventing its degradation and cytosolic oxidation. In a similar cell model, the adrenal chromaffin cell, our and other groups have directly

measured the free cytosolic catechols (10-100 µM) whereas the vesicular content extremely high (0.8-1 M, depending on the animal species studied) (Montesinos et al., 2008).

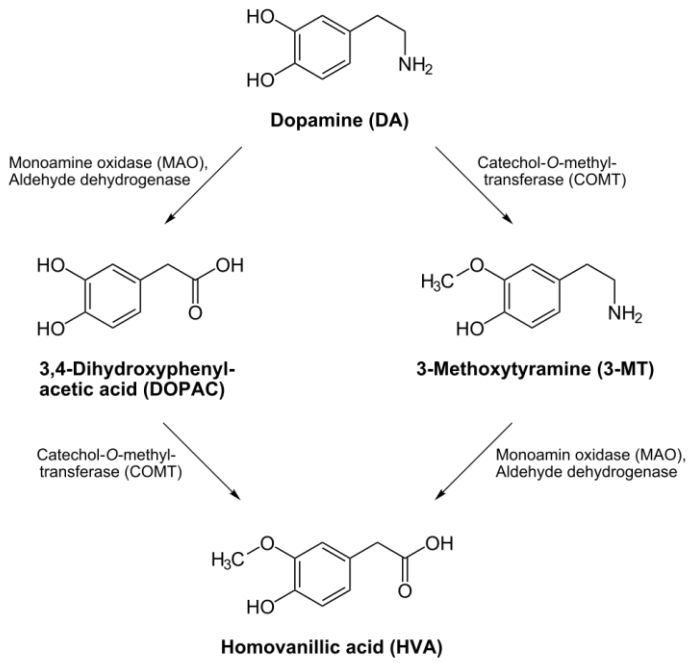

Fig. 01. DA downgrading. Both degradation pathways involve the enzymes MAO and COMT, albeit in reverse order: MAO catalyses dopamine to DOPAC, and COMT, DOPAC to HVA; whereas COMT catalyses dopamine to 3-MT and MAO, 3-MT to HVA (modified of Eisenhofer et al., 2004).

Being the enormous gradient formed across the vesicular membrane (>10,000) if DA uptake by the secretory vesicles is not optimum, high cytosolic levels of DA would cause cell toxicity and prompt neuronal death. During these last three decades it has been hypothesized that DA and its oxidation metabolites, along with high-cytosolic-calcium levels and α-synuclein, compose a toxic mixture in PD, especially in axon terminals and dendrites. This causes a progressive neuronal loss, long before clinical manifestations (Bisaglia et al., 2007; Zucca et al., 2017).

2. PLATELETS AS NEURONAL MODEL

2.1. PLATELETS

Platelets are small (2-3 µm diameter) anucleate blood cells derived from bone marrow megakaryocytes, whose half-life is around 8-12 days (Blair & Flaumenhaft, 2009). Among their main functions, we might highlight the role they play in haemostasis either thrombosis, inflammation, angiogenesis, anti-microbial host defence or mitogenesis

(Fitch-Tewfik & Flaumenhaft, 2013). Platelets are indeed a natural source of growth factors, such as PDGF (platelet derived growth factor), TGF-β (transforming growth factor) as well as being powerful chemotactic agents (Blair & Flaumenhaft, 2009).

2.2. SECRETORY GRANULES OF PLATELETS

Platelet granules are unique among all kind, both in its content and in its life cycle. They essentially contain 3 types of granules (**Fig. 02**), including α-granules, dense core granules and lysosomes. These last ones have not been well studied, but they have many acid hydrolases and cathepsins, serving a role in endosomal digestions (Fitch-Tewfik & Flaumenhaft, 2013; Sharda & Flaumenhaft, 2018).

α-Granules are far and wide the largest in number, compounding a 10% of platelet volume. They contain a great variety of membrane proteins and soluble cargo, released by SNARE protein family, in response to various stimuli (Piersma et al., 2009).

Dense granules are comparatively 10-fold less abundant than α-granules. They have high concentrations of cations, polyphosphates, adenine nucleotides and bioactive amines, such as histamine and serotonin (5-HT), enclosed inside (Sigel & Corfu, 1996).

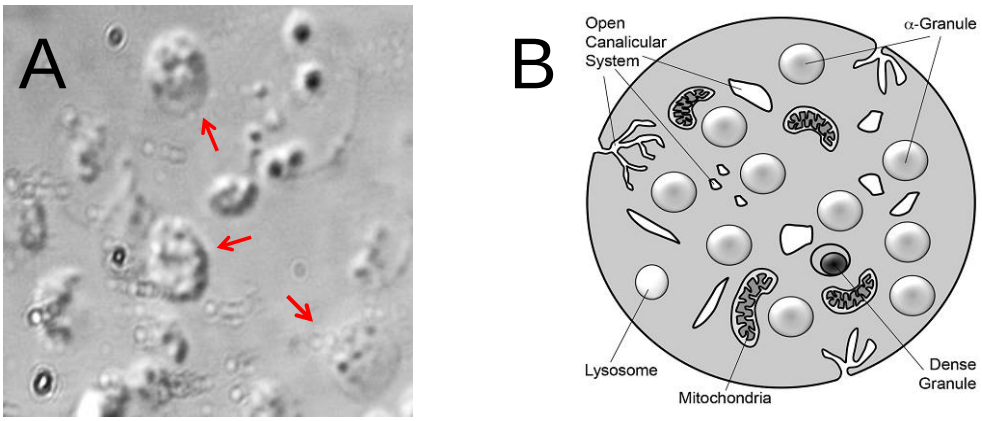

Fig. 02. Platelet components. Panel A shows a bright field microscopy image showing a set of platelets (red arrows). In panel B, the different subcellular compartments of a platelet are schematically represented, including the α-granules and the dense core granules responsible for 5-HT accumulation (modified of Fitch-Tewfik & Flaumenhaft, 2013).

The uptake, accumulation and release mechanisms of soluble compounds in the secretory granules of platelets are very similar to the ones observed in neuroendocrine cultures, like

chromaffin cells or dopaminergic neurons although no synthetic pathways seem to occur in platelets being all 5-HT uptaken from plasma (**Fig. 03**).

The accumulation of many biological amines from cytosol to the interior of secretory granules is made through VMAT (Vesicular Monoamine Transporters). Two isoforms fo VMAT have been described (VMAT1 and VMAT2).

This uptake system uses the proton gradient engendered by the action of V-ATPase (proton Vesicular ATPase). In fact, both VMAT and V-ATPase are not exclusively found in enterochromaffin cells or neurons, but also in platelets (through VMAT2). This transporter is the responsible of 5-HT accumulation inside platelets secretory granules. In the granules of these cells, there is also another system, VNUT (Vesicular Nucleotide Transporter), which concentrates ATP in the vesicle (Brunk et al., 2006; Flitch-Tewfik & Flaumenhaft, 2013; Moriyama et al., 2017).

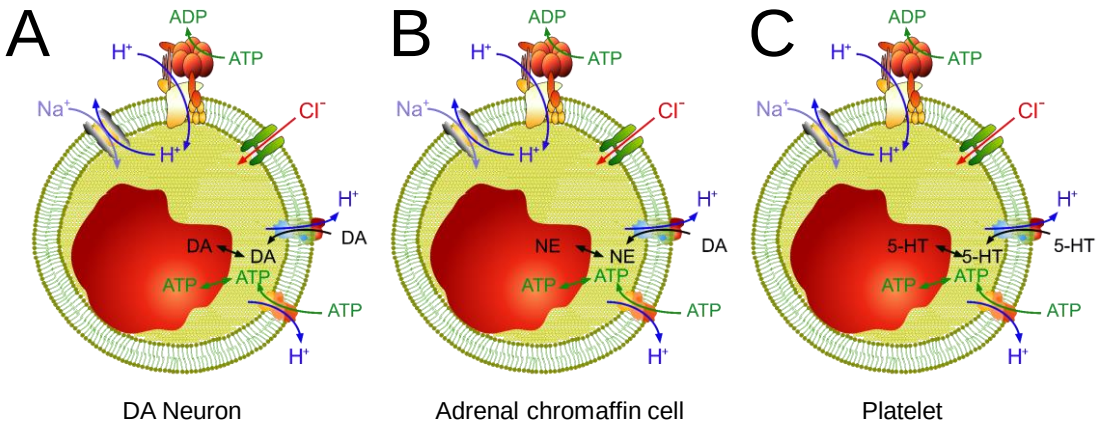

Fig. 03. Comparative diagram of the different secretion granules. Functionally, the dense granules of the platelets (panel **C**) have similarities with the dense core granules of the neuroendocrine cell types (panel **B**) and dopaminergic neuron secretory vesicles (panel **A**). VMAT system is responsible for catechol and indoles uptake, in collaboration with the H + V-ATPase. Other systems shown are the VNUT, responsible for the uptake of ATP and various ion exchangers and transporters systems (modified of Fitch-Tewfik & Flaumenhaft, 2013).

Molecular bases of granule secretion in platelets have not been studied enough. As chromaffin cells, platelets accumulate high concentrations of bioactive amines and peptides ready for release. Alteration in the releasing dynamic of 5-HT from platelets may reveal the dysfunctions that would be common to other amine-storing cells including neurons.

3. PREVIOUS RESULTS

Last year Angelica Figueroa (a former student from this Medical School) performed a project in our laboratory, which consisted in implementing the methods for isolation functional human platelets.

Her aim was focus on set an effective protocol for the isolation, quantification and functional maintenance of platelets, likewise determine 5-HT concentration and evaluating platelet dense core granule uptake and accumulation capacity of catechol and indoles, made through high-pressure liquid chromatography coupled to with electrochemical detection (HPLC-ED).

From that study she obtained the following conclusions:

- Using a relatively small volume of blood, it can be obtained a significant number of healthy human platelets, enough for studying the secretion process.
- She was success in purifying platelets to over 90%, which were functionally preserved along several days in optimal conditions.
- Isolated platelets were able to uptake 5-HT, L-DOPA and 5-OH tryptophan, but they do not have the enzymatic machinery to transform L-DOPA to dopamine or 5-OH tryptophan into 5-HT, due to the lack of LAAD enzyme.
- Platelets release 5-HT through an exocytotic mechanism that can be monitored by amperometry, from both single and platelet population.
- Functional characterization of the secretory pathway in human platelets brings a wide range of possibilities in the diagnosis of conditions that involve any disturbance in this route. (Figueroa et al., 2018).

Altogether, she set a precedent for the current investigation. With the development of the present study we are trying to take a step beyond.

AIMS OF THE STUDY

The biosynthesis of dopamine (DA) by dopaminergic neurons is possible thanks to the hydroxylation of L-tyrosine to L-DOPA, by tyrosine-hydroxylase (TH), follow by its decarboxylation by means of aromatic-L-amino acid decarboxylase enzyme (LAAD). Then, DA is brought into secretion granules by vesicular monoamine transporter (VMAT). This uptake mechanism can be deregulated due to diverse environmental and genetic factors, so that DA accumulation in granules is altered, and therefore, its cytosolic concentration is increased.

One of the theories to explain the genesis of Parkinson's disease (PD) is related to neurodegeneration caused by the toxicity of DA and its main metabolite, DOPAC, for being free at the cytosol, instead of contained into the secretion granules of this nigrostriatal dopaminergic neurons (Bisaglia et al., 2007; Zucca et al., 2017).

It has been observed that these granules, in both neurons and platelets, share analogous mechanisms for the uptake of catechol and indoles, respectively. For that reason and due to the impossibility of using human neurons or neuronal cultures, the functional serotonin (5-HT) storage from purified human platelets could be a valuable correlation for future early diagnosis of PD, long before clinical manifestations appear.

Our hypothesis is that in patients suffering from PD, due to the neurodegeneration, there is a meaningful decrease of both catechol and indoles concentration, which would not happen in non-PD people.

Having this in mind, the main aims of this study are established below:

1. To optimize the isolation, quantification and functional maintenance protocol, used in the previous study one year ago.
2. To determine basal 5-HT concentration and evaluate, above all, the uptake capacity, even though it is also possible to analyse the response of platelets in experiments of 5-HT release, depletion and inhibition by drugs. For these measurements we will use high-pressure liquid chromatography coupled with electrochemical detection, (HPLC-ED).
3. To compare the results of uptake capacity obtained from, supposedly volunteers, and to establish if there are possible significantly threshold values between them.

MATERIAL AND METHODS

1. BLOOD SAMPLING

Human platelets were obtained from blood samples in *Acid Citrate Dextrose* anticoagulation solution (ACD) from random donors of the Servicio de Neurología del Hospital Universitario de Canarias (HUC), Centro Neurológico del Dr Antonio Alayón and the Facultad de Ciencias de la Salud (Medicine Section) of Universidad de la Laguna.

All subjects were informed about the aim of the study and procedure. All relevant information such as personal and familial diseases was included in an anonym clinical form (see **Appendix 01**).

Two main procedures were performed:

1) Complete study (measuring total serotonin content and the serotonin content after incubation with increasing concentration of exogenously added 5-HT to evaluate the uptake) was made with PD and age-matched volunteers and

2) Screening test (quantifying only basal 5-HT content and its uptake when incubated with 10µM 5-HT) This was intended only to young medical students.

Both methods mainly differ principally in the amount of blood sample required and experiment duration (the screening procedure can be performed in 1 day, whereas the full study requires twice long).

2. PLATELETS MANAGEMENT

2.1. PLATELET ISOLATION AND STORAGE

Platelet isolation protocol used in the current work is a modification of various standardized protocols (Abcam; Ge et al., 2011), implemented by Dr Pablo Montenegro. The different steps followed are described below. All the steps have to be done carefully at 25-30ºC in order to prevent platelet activation:

1. Peripheral blood samples (9-10 mL for complete study, or 4 mL for the screening procedure) were extracted by venepuncture (VACUTEST KIMA® K2-EDTA). Blood sample tubes were gently flipped and left for 15 min in horizontal position.

2. Blood sample tubes were centrifuged in a vascular oscillating rotor centrifuge during 20 min at 200xg with no-brake. After spin, 2/3 of the platelet-rich plasma (PRP) was carefully collected with a Pasteur pipette.

3. The PRP supernatant was diluted 1:1 in HEP Buffer (in mM). NaCl (140), KCl (2.7), EGTA (5), HEPES (3.8), pH 7.4 (NaOH). Prostaglandin E1 was added (1 µM final concentration) to avoid platelet activation. Samples were mixed gently by inverting and centrifuged for 15 min at 100xg with no-brake.

4. The supernatants were gathered into the corresponding sterile conic tube, with a Pasteur pipette, and centrifuged it during 20 min at 800xg with no-brake.

5. Platelet pellet samples were collected and washes twice with 1 mL (complete study) or 500µL (screening) of Platelet Wash Buffer (in mM) NaCl (150), EDTA (1), Glucose (50), Na^+-Citrate (10), pH 7.4 (NaOH).

6. Washed platelet pellets were resuspended in 4 mL (complete study) or 1mL (screening) of SSP+ medium supplemented with 5 mM D-Glucose and antibiotics (in mM) NaCl (69.3) NaCl, KCl (5), $MgCl_2$ (1.5), Na_2HPO_4/NaH_2PO, (28.2), Na^+-Citrate (10.8), Na^+-Acetate, (32.5), pH 7.2 (NaOH), and maintained in Falcon tubes (Tubespin® bioreactor, TRP). Prostaglandin E1 and ascorbic acid were added (1 µM/10 µM final concentration) to avoid platelet activation, and 5-HT oxidation respectively.

Isolate platelet samples were incubated at room temperature with gas exchange and non-stopping rotation (25 rpm, 45°) to prevent platelets from aggregating. By means of this protocol, platelets were viable for a 7-10 days period. It is important to maintain a physiological pH 7.2-7.4 because previous studies confirm that alkaline pH has a significant effect on the dense granules' secretion, leading to platelet activation (Ge et al., 2009).

2.2. TURBIDIMETRY ANALYSIS

In order to normalize the amount of platelets present in each sample, we had performed a search for a rapid and reliable method for quantification. We had compared the total

proteins, platelet counting in a haemocytometer, flow cytometry, particle counting assay and turbidimetry. This latter method was finally chosen.

The standard isolation protocol described yields ≈1x10^5 platelets/µL. This platelet concentration has been established as the optimum for later experiments (Ge et at., 2009; Haynes et al., 2010; Haynes et al., 2011).

Turbidimetry technique consists of measuring the loss of intensity of transmitted light, due to scattering effect of particles suspended in a buffer. Monochromatic light passes through the cuvette that contains the platelets solution. A photoelectric cell collects the light and determinates the quantity of absorbed light, which is proportional to suspended platelet concentration (Vasudevan, 2010) (**Fig. 04**).

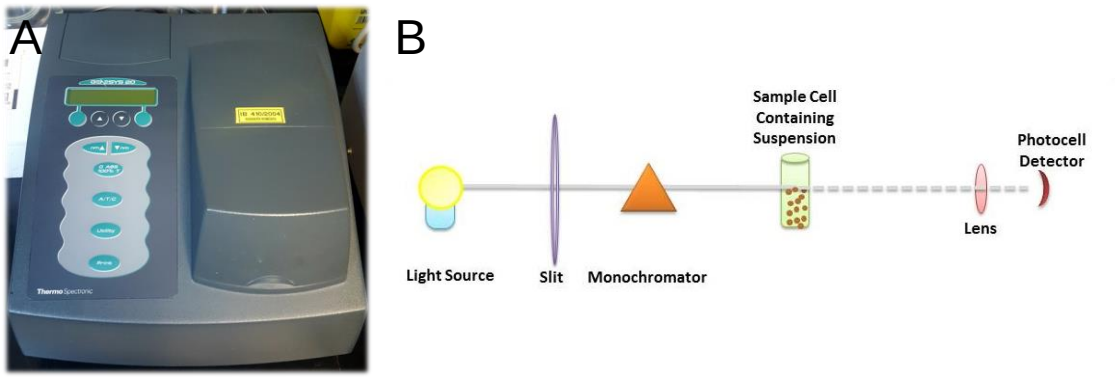

Fig. 04. Scheme of a turbidimeter. Panel A shows a picture of the spectrophotometer used in the present study. Panel B represents sequentially the different components of the turbidimetry measurements principle (modified of Heda, 2013).

All concentration measurements we performed in a GENESYS^{TM} 20 spectrophotometer (ThermoSpectronic). We used 600 nm excitation wavelength to determine platelet concentration. One hundred µL of isolate platelet suspension was diluted in 1mL SSP+ medium (1:10). As blank solution we used SSP+ solution (see **Appendix 02**). After performing the measurements, the dilution volume was corrected to the final platelet concentration desired.

3. DETERMINATION OF BASAL 5-HT AND UPTAKE

3.1. 5-HT UPTAKE PROTOCOL

Figure 05 shows the procedure optimized for 96-well culture plates. Serotonin uptake experiments, carried out by incubation of the platelets in different media, following the protocol (**Fig. 05**):

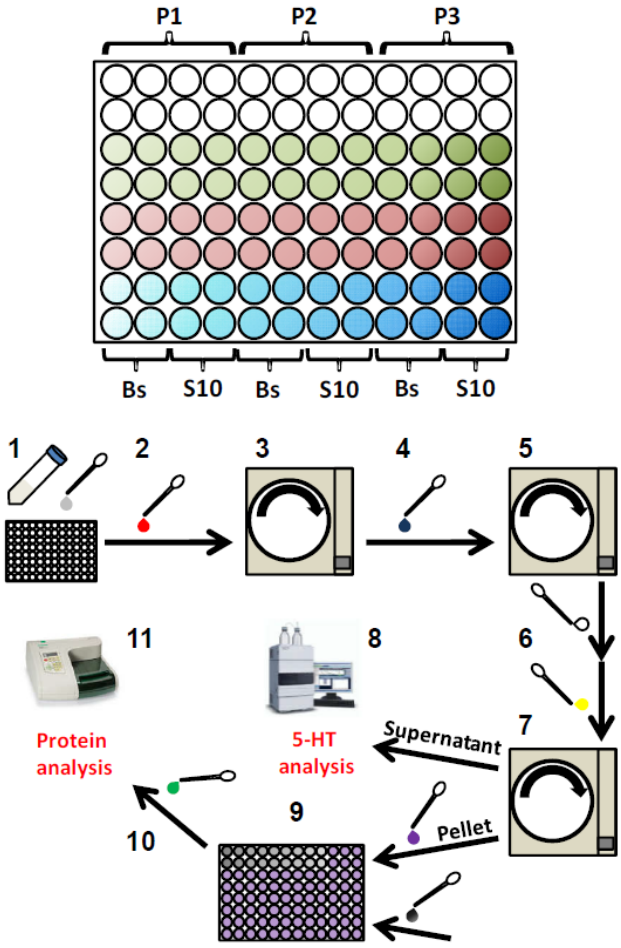

Fig. 05. Uptake protocol scheme. All the numbered steps are described below. Duplicates were made from each sample, for both complete study and screening procedures.

1. 100 µL of platelet suspension were placed into a 96-conical-bottom-well plate (Sarstedt).

2. 100µL of 5-HT solutions (0-1000 µM final concentration) were gently added to the assay buffer containing the platelets solution (in mM) NaCl (69.3), KCl (5), $MgCl_2$ (1.5), Na_2HPO_4/NaH_2PO_4 (28.2) Na^+-Citrate (10.8), Na^+-Acetate (32.5), Glucose (5), ascorbic acid (0.2), 5-HT, pH 7.2 (NaOH). The plate was incubated for 2 h at 20-25 ºC, with no light and gentle agitation.

3. The plate was the cooled at 0-4ºC and centrifuged at 3,200xg at 4ºC during 10 min.

4. The supernatant was taken away, the resulting pellet was washed with 200 µL of clean SSP+ solution.

5. The plate was again centrifuged at 3,200xg at 4ºC for 5 min.

6. The resulted supernatant was drawn back and the pellet from the plate wells is smoothly resuspended avoiding creating bubbles, using 25 µL of milli-Q water. Thereupon, 200 µL of a perchloric acid (PCA) solution was added (0.05625 N PCA, 337.5 µM L-cysteine, 337.5 µM EDTA and 225 nM isoproterenol). Anew, the plate was cooled at -80ºC.

7. The plate was defrosted up to 0-4ºC and centrifuged at 3,200xg at 4ºC for 10 min.

8. For the 5-HT analysis, 150 µL of the supernatant were passed to HPLC vials for analysis.

9. For the protein quantification, the pellet was gently resuspended, avoiding making bubbles, in 50 µL of a solution made of 0.1M NaOH and 5% SDS. Next, 25 µL were carefully moved to a 96-flat-bottom-well plate (Nunclon™ Delta Surface, ThermoScientific). Then 25 µL bovine serum albumin (BSA) solutions were used as reference standard. BSA was diluted in 0.1M NaOH, 5% SDS for performing a calibration curve (0-2 mg BSA).

10. Protein analysis was performed using the bicinchoninic method. 4% g/L $CuSO_4$ was diluted in bicinchoninic acid following the procedure described by the manufacturer. The well plate was gently agitated and incubated at 37ºC without light for 30-45 min.

11. Protein concentration was determined using a spectrophotometer plate reader (iMark microplate reader, Biotek instruments Inc, Winooski, USA), using a wavelength set to 590 nm.

3.2. HPLC-ED

Samples of 150 µL were transferred to micro-vials (**Fig. 06**).

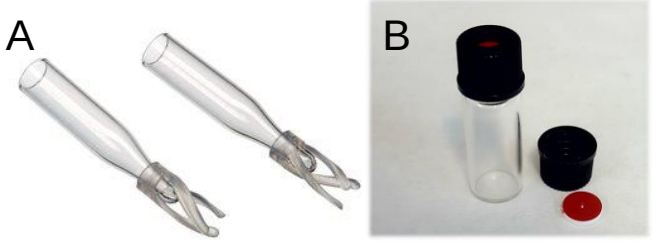

Fig. 06. Vials. Picture A shows the clear glass conical inserts, with a bottom spring (ThermoScientific), while picture B corresponds to a vial, with its filter and cap (Scharlab).

The HPLC system (**Fig. 07**) used a refrigerated-rack automatic injector (SIL-6B model, Shimadzu). Separation was made at 1 mL/min flow using a 12.5 cm-length column (Analytic Tracer, Teknokroma, Barcelona) filled in with C-18 Spherisorb ODS-2, with a 5 µm particle size.

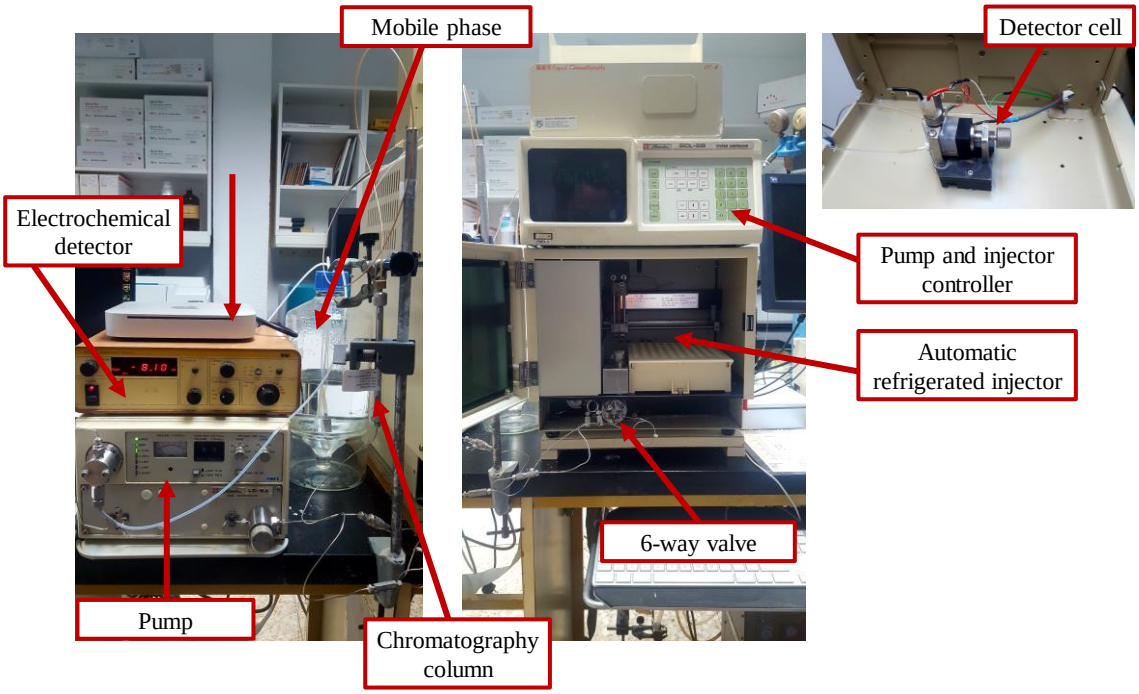

Fig. 07. HPLC-ED. Scheme of the different parts of chromatographer used in the present work.

The mobile phase used was a phosphate buffer (9.66 g/L NaH_2PO_4, 3.72 mg/L EDTA, 7-10% methanol (v/v), pH 4). The mixture was previously filtered through a 0.22 µm filter (Millipore) and degassed by bubbling with He.

Electrochemical detection was performed at +700 mV using an electrochemical detector (LC-4B model, Bioanalytical Systems, West Lafayette, USA).

Data acquisition was performed using an USB ADDA card (6008 National Instruments) and sampled at 1 Hz to Mac Mini Apple computers using a tailoring-made acquisition program written in LabView 11 by Dr Yezer González. External standards were made by 200 nM of 5-HT and 200 nM isoproterenol, the latter was used as the internal standard.

3.3. RESULTS ANALYSIS

HPLC-ED data were analysed using a locally written macro performed by Dr Miguel A. Brioso in IGOR-Pro (Wavemetrics, OR, USA) (**Fig. 08**).

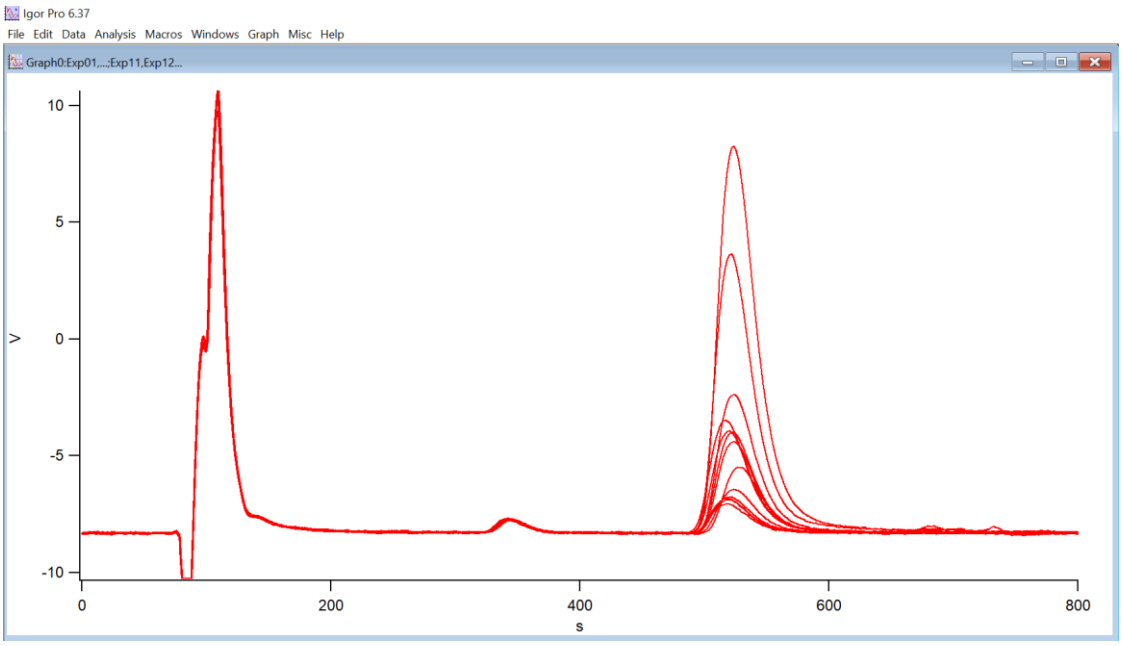

Fig. 08. Chromatograms. This is an IGOR-Pro view of overlapping chromatograms, where different picks (voltage versus time) can be seen. Sequentially it is shown (retention time) i) front peaks corresponding to solvents, ii) isoproterenol (200 nM, internal standard) and iii) serotonin. Note the different size of 5-HT peaks. As amplifier gain was 1 nA/V the ordinate scale is also in nA).

For converting the integrated current (nano Coulombs, nC) into concentration we used:

$$[5\text{-}HT]\ (nM) = \frac{5\text{-}HT\ area(nC)\ \ x\ \ [Isoproterenol]\ (nM)\ \ x\ \ CF}{Isoproterenol\ area\ (nC)}$$

Where CF is the *correction factor*, obtained from external standards. The 5-HT measured sample concentrations were normalized to the protein concentration as mentioned.

4. STATISTICS

All the graphic and statistical analyses were done using Prism 5 program (GraphPad) (California, USA). Data were represented as means ± SEM. Statistical analyses of variance (ANOVA), student t-test or other non-parametric test were assigned to a level of significance as * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

5. MATERIALS

The water was purified in an Elix10/MilliQGradient A-10 system from Millipore (Darmastadt, Germany). Serotonin and isoproterenol were supplied by Merck (Darmastadt, Germany). Bicinchoninic solution was supplied by Sigma-Aldrich (St. Louis, MI, USA). Perchloric acid was provided by Panreac (Barcelona, Spain). Prostaglandin E1 was provided by Santa Cruz Biotechnology (Texas, USA). Unless otherwise indicated, other salts and reagents were purchased from Sigma-Aldrich or Merck.

RESULTS AND DISCUSSION

1. PLATELETS PROTOCOL

In our laboratory we have re-established a platelet isolation protocol minimizing considerably the presence of other cellular components of PRP, such as erythrocytes and leukocytes. In fact, in the present study this protocol has been and optimized performing a better and faster platelet-counting protocol using turbidimetry measurements, than classic hemocytometer analysis.

On another hand, platelets maintenance medium has consisted in clinic-used SSP+ solution supplemented with 5 mM D-Glucose, 1 µM prostaglandin E1 and 10 µM ascorbic acid. This new medium is slightly hypertonic (320-350mOsm/L) in comparison to natural extracellular medium (around 300mOsm/L), minimizing serotonin (5-HT) passive diffusion out from platelets as well as preserving this indole to oxidize (Robert & Johnson, 1988). Finally, addition of prostaglandin E1 avoids platelet activation and release of granule cargo.

2. OPTIMIZATION OF EXTRACTION-MAINTENANCE [5-HT] PROTOCOL

One challenging issue was to establish not only an optimized 5-HT extraction protocol from human platelets but to minimize the degradation of this indole prior to HPLC-ED determination. According to literature, 5-HT is stable at a pH range 2-6 whereas high temperature and light increase the degradation rate. Also related to this, previous 5-HT extraction protocol implemented TENT (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% TritonX, pH7.4 NaOH) used for platelet pellet resuspension. This detergent solution increases the adsorption of 5-HT samples to the vial glass.

First of all, TENT was replaced for H_2O–PCA (perchloric acid) solution for the resuspension step. This PCA solution (see Material and Methods) contains an optimal pH

range and preservative agents that increase 5-HT stability (Thorré et al., 1997; Audhya et al., 2012).

Altogether with maintenance of the cold chain through the use of refrigerated systems contributed to the enhancement of HPLC-ED results (in **Appendix 03** are showed the effect of PCA and TENT in basal and 10µM 5-HT uptake quantification).

3. DETERMINATION OF [5-HT] IN COMPLETE STUDY ASSAYS

At least, two groups of subjects, Control and Parkinson's disease (PD) Patients, previously clinical-diagnosed were established (Servicio de Neurología, Hospital Universitario de Canarias (HUC) and Centro Neurológico Dr Antonio Alayón). **Complete study** experiments implicate full-doses-dependence 5-HT platelet uptake. In **Fig. 09A** are represented both controls and PD patients, showing normalized per proteins total 5-HT concentration ($[5\text{-HT}]_T$) related to different 5-HT concentrations. Both curves present a first slow rate 5-HT uptake that gradually increments up to a stationary plateau, around a 10 µM [5-HT] incubation, finally exponentially incrementing when doses reach 100 µM [5-HT]. At a first sight, it can be assumed that the last step could correspond to unspecific 5-HT accumulation, probably in other compartments different from proper platelet secretory granules. Despite both curves present the same behaviour, there are significant differences between the two groups of subjects in each point that leads the full-doses-dependence curves.

Fig. 09B shows a concrete representation of basal $[5\text{-HT}]_T$ and 10 µM $[5\text{-HT}]_T$ for each group. PD's patients compared to control group obtained significantly minor values in both cases, **basal** (Ctrl: 2.19 ± 0.14 nmol|mg protein|$^{-1}$, n=25; Park: 0.30 ± 0.03 nmol|mg protein|$^{-1}$, n=13; mean ± SEM) and **5-HT 10µM** (Ctrl: 7.25 ± 0.54nmol|mg protein|$^{-1}$, n=25; Park: 4.33 ± 0.54 nmol|mg protein|$^{-1}$, n=13).

Moreover, **Fig. 09C** represents each value for both groups showing no overlap of points between Control and PD patients in basal $[5\text{-HT}]_T$ measures, despite some overlapping points in 5-HT10µM ones.

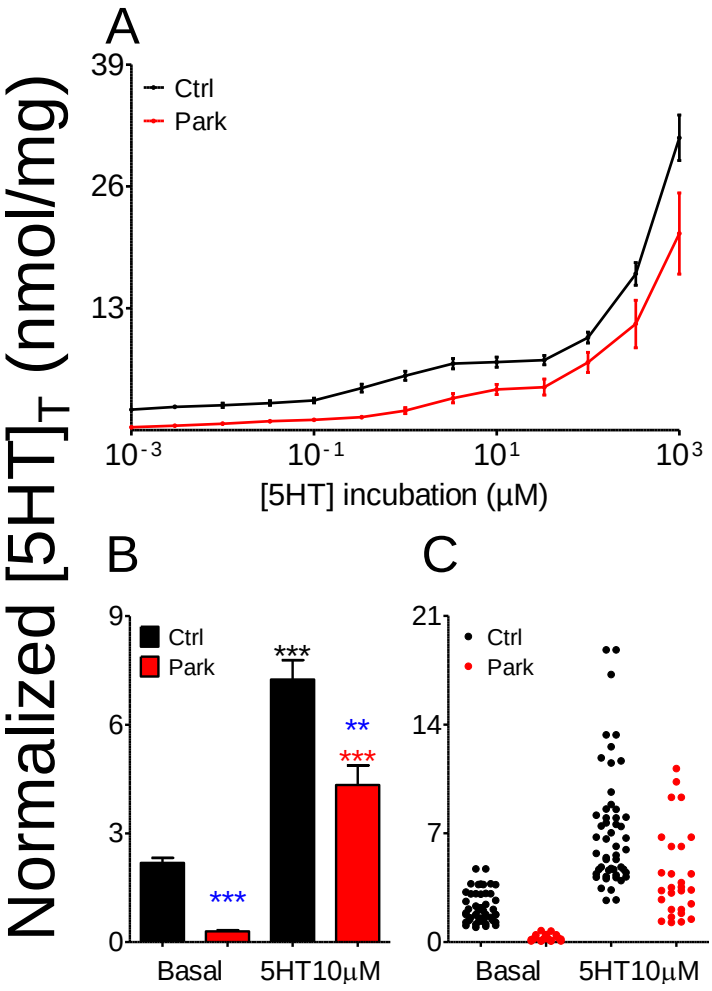

Fig. 09. 5-HT accumulation measurements in platelets of PD patients (Park) and control (Ctrl) groups. Human platelet samples were incubated with different concentrations of 5-HT for 2h and then processed to obtain $[5\text{-HT}]_T$, measured by HPLC-ED (see Material & Methods). Upper panel A shows dose-dependent accumulation of 5-HT in human platelets, observing control study groups (black) and PD patients (red). Panels B and C reflect the basal 5-HT accumulation data and after incubation with 5-HT10 µM for both study groups in average values (panel B) or each individual subject (panel C). The statistical analysis of the dose-dependent uptake curves was carried out through the ANOVA test, while the basal and 5-HT10 µM data were compared using the Kruskal-Wallis test. The significance levels for internal control- and PD groups values are represented in black and red respectively, while the blue one shows comparison between groups for each measurement (* $p<0.05$, ** $p<0.01$, *** $p<0.005$).

Summarizing the main hypothesis of the present study, it has been postulated that deregulations in the uptake and accumulation of 5-HT in dense granules of platelets could reflect the behaviour in dopaminergic neurons.

According to the theory of neurotoxicity this deregulation would lead to a decompartmentalization of dopamine, this neurotransmitter and its metabolites being stored in the cellular cytosol, leading to neurodegeneration in the non-clinical phase (Bisaglia et al., 2007; Zucca et al., 2017). Since practically all of the 5-HT present in platelets accumulates preferentially in dense granules (Ge et al., 2011), these basal $[5\text{-HT}]_T$ results could establish a threshold from which a subject would reveal a non-clinical manifestation of suffering from PD in the future.

Experiments incubating platelets with 5-HT 10 µM results values could be taken as a secondary indicator of the accumulation capacity of 5-HT in platelets, compared with the previous basal levels. In fact, an empirical correlation of these data could be established to improve the future diagnosis, although such a resolution will not be contemplated in the present work.

4. DETERMINATION OF [5-HT] IN SCREENING ASSAYS. ITS FUTURE AS DIAGNOSTIC TOOL

The results obtained with PD patients previously clinically diagnosed have reflected significant differences for basal levels of 5-HT accumulation, as well as the capacity of uptake and accumulation of this indole in human platelets. As a future diagnostic tool, we have verified the robustness of the previous data by comparing them with new data obtained from theoretically healthy volunteers belonging to students and staff of the Facultad de Ciencias de la Salud (Medicine Section) de Universidad de la Laguna (ULL). In the so-called **screening** assays, two rounds of 28 volunteer subjects were carried out where, after the isolation of platelet samples, only the basal $[5\text{-HT}]_T$, as well as indole 5-HT 10 µM-incubation uptake capacity was determined. Samples were named only using the last three numbers plus the letter of their identity card, the same as the clinical form they had to fill in. The results from the first round of 18 volunteer can be seen in **Fig. 10**. As observed, practically all the subjects presented significant higher basal [5-HT] levels with respect to values recorded in PD patients, without observing significant differences in comparison to the control data.

However, three subjects appeared with values that are not representative of either group. From those, one presented a 5-HT 10 µM uptake significantly superior to the PD referents, while the other two again presented values outside those established for control and PD.

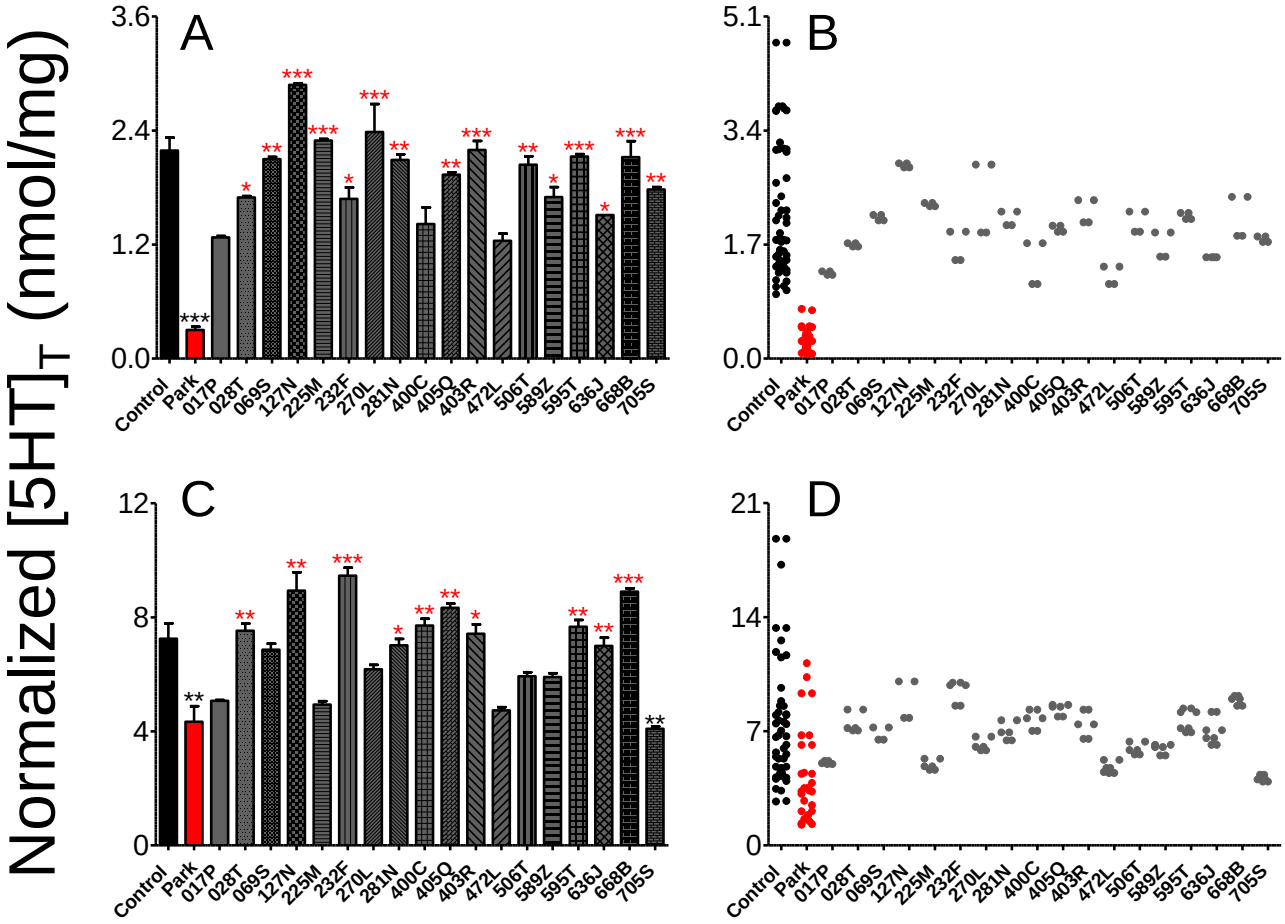

Fig. 10. 5-HT accumulation measurements in platelets of first round volunteers. Human platelet samples were incubated with different concentrations of 5-HT for 2h and then processed to obtain $[5\text{-HT}]_T$, measured by HPLC-ED (see Material & Methods). Upper panels show the average (A) and particular (B) data obtained from the basal 5-HT measurements of each voluntary subject (gray) compared to the control data (black) and PD patients (red) while the lower data represent their analogues for uptake after 5-HT 10 µM incubation. The statistical analysis of basal and 5-HT 10 µM data were compared using the Kruskal-Wallis test. The significance levels between control and PD are represented in black and red respectively (* $p<0.05$, ** $p<0.01$, *** $p<0.005$).

Notice that the overall statistical significance in the uptake data was lower than their basal correspondents, with a greater dispersion for each individual subject. Even one subject presented a 5-HT uptake within the values corresponding to PD, although it was considered in the range of controls by their basal 5-HT measurements.

These results again show the need to establish an empirical correlation between basal data and 5-HT 10 µM uptake. After these results, however, it was considered to include all voluntary subjects within the control group, although those already mentioned for future analysis are taken into consideration. The inclusion of these data allowed the elaboration of new dose-dependent uptake curves, used for the second round of voluntary subjects already mentioned (**Fig. 11**).

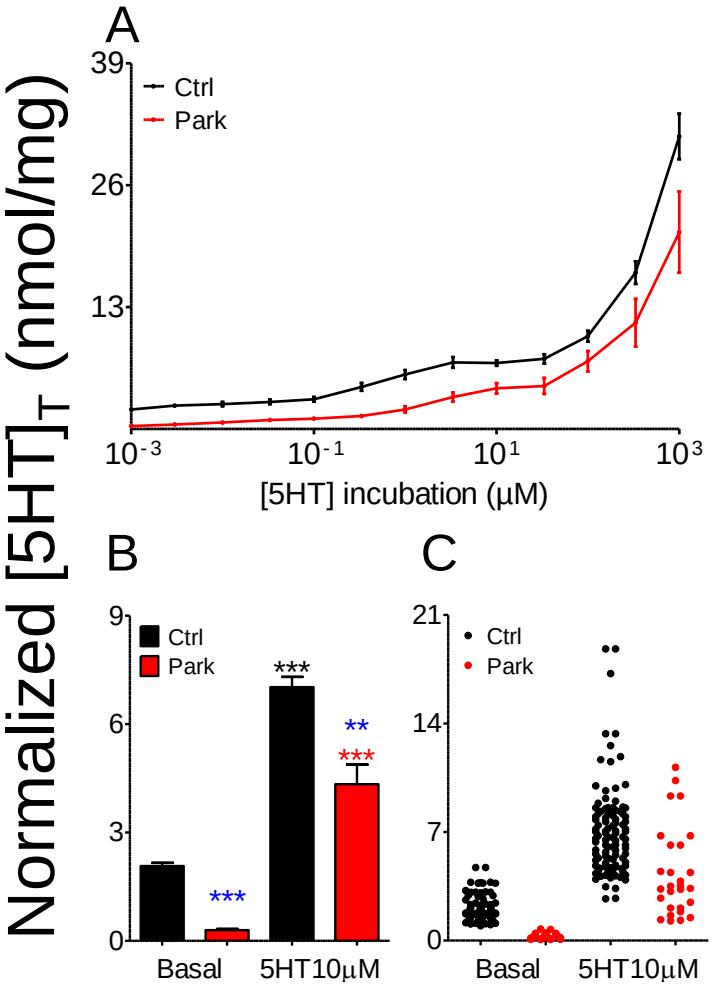

Fig. 11. 5-HT accumulation measurements in platelets of PD patients (Park) and control (Ctrl) groups after including first round of screening donor. Human platelet samples were incubated with different concentrations of 5-HT for 2h and then processed to obtain $[5\text{-HT}]_T$, measured by HPLC-ED (see Material & Methods). Upper panel A shows dose-dependent accumulation of 5-HT in human platelets, observing control study groups (black) and PD patients (red). Panels B and C reflect the basal 5-HT accumulation data and after incubation with 5-HT10uM for both study groups in average values (panel B) or each individual subject (panel C). The statistical analysis of the dose-dependent uptake curves was carried out through the ANOVA test, while the basal and 5-HT10µM data were compared using the Kruskal-Wallis test. The significance levels for internal control and PD groups are represented in black and red respectively, while the blue one shows comparison between groups for each measurement (* $p<0.05$, ** $p<0.01$, *** $p<0.005$).

Again, PD patients compared to control group obtained significantly minor values in both cases, **basal** (Ctrl: 2.08 ± 0.09 nmol|mg protein|$^{-1}$, n=43; Park: 0.30 ± 0.03 nmol|mg protein|$^{-1}$, n=13; mean ± SEM) and **5-HT 10 µM** (Ctrl: 7.02 ± 0.28 nmol|mg protein|$^{-1}$, n=43; Park: 4.33 ± 0.54 nmol|mg protein|$^{-1}$, n=13), with no overlapping in 5-HT basal data. Finally, for the second round, 10 volunteers participated in the study, whose results can be seen in **Fig. 12**. In this group of subjects, the differences regarding the counting of PD patients were even more marked. However, again, the values of 5-HT 10 µM uptake were less robust, comparing basal [5-HT] ones.

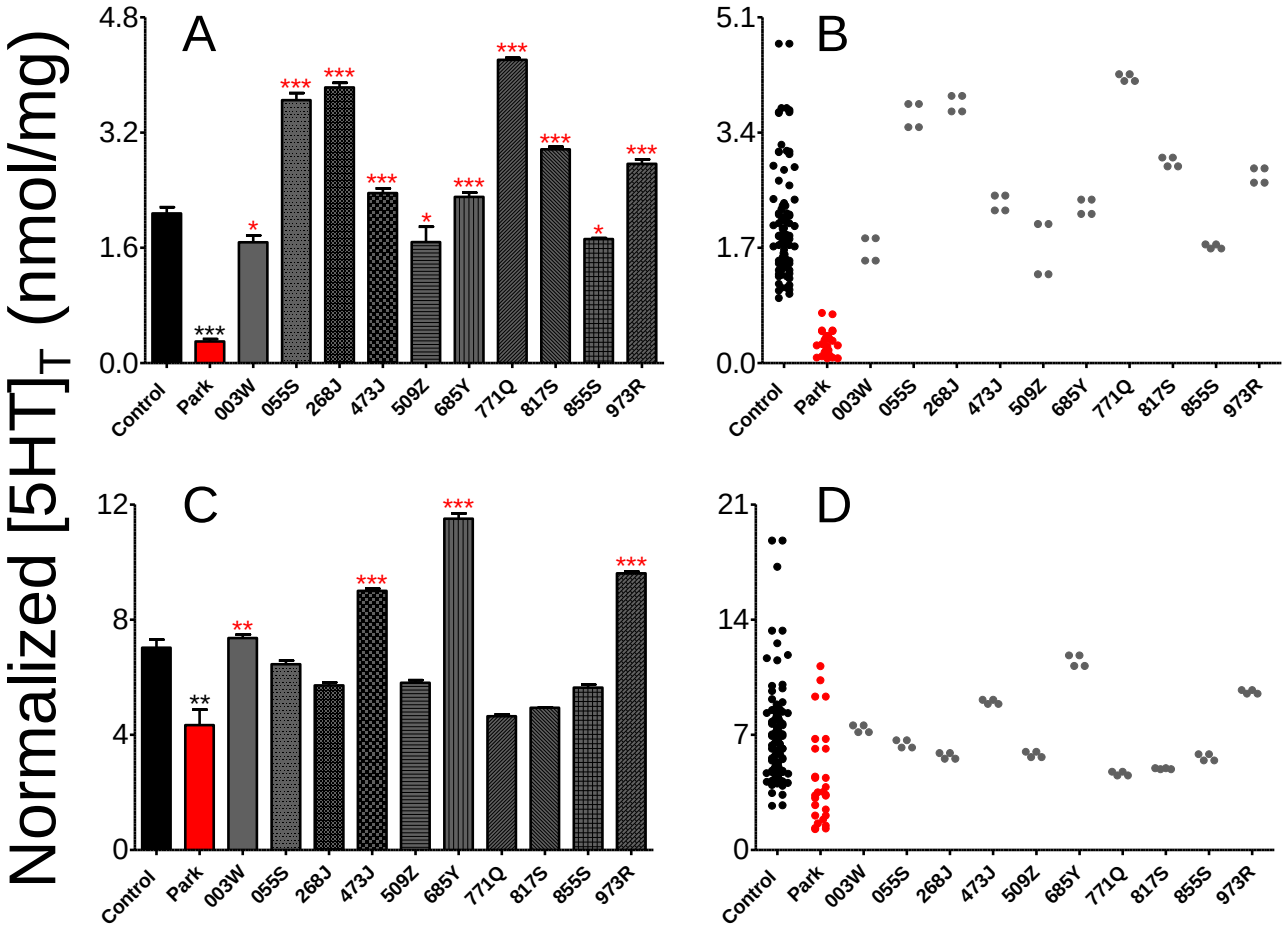

Fig. 12. 5-HT accumulation measurements in platelets of second round volunteers after including first round screening donor. Human platelet samples were incubated with different concentrations of 5-HT for 2h and then processed to obtain $[5\text{-HT}]_T$, measured by HPLC-ED (see Material & Methods). Upper panels show the average (A) and particular (B) data obtained from the basal 5-HT measurements of each voluntary subject (gray) compared to the control data (black) and PD patients (red) while the lower data represent their analogues for uptake after 5-HT 10 µM incubation. The statistical analysis of basal and 5-HT 10 µM data were compared using the Kruskal-Wallis test. The significance levels between control and PD groups are represented in black and red respectively (* $p<0.05$, ** $p<0.01$, *** $p<0.005$).

5. FUTURE PERSPECTIVES

In order to increase the internal and external validity of the method, it is vitally important to increment the sample size, the N for the experiment. This may also help to establish more precisely [5-HT] range for both groups. Currently, our group is going to extend the cohort of PD patients to the HUNSC and to the Association of PD patients of Tenerife.

Also currently, as previously mentioned in the objectives section, our group have been developed platelet 5-HT released, (through thrombin assays), and inhibition of 5-HT uptake pathways protocols in order to provide more information between both study groups and further elucidate the possible neuronal behaviour in PD patients. Although promising results are being obtained, they have not been considered in the present work.

In this line, the only way to ensure that the results obtained are reliable is by following all the patients and volunteers in time, like in a prospective study, to see if [5-HT] are related to future appearance of PD.

Our group has applied for a patent for this method as a diagnostic tool. As HPLC is not an easy technique for handling large number of samples and cannot easily implemented in clinical laboratories, we are starting conversation with investors for developing diagnostic kits based either in ELISA or (better) in chemiluminescence analysis of 5-HT that could be used in hospital core laboratories to quantify platelet serotonin. These goals open an exciting field of future research and innovation although this future is currently out of the scope of the current TFG.

CONCLUSIONS

As it is discussed in the current work, we can reach the following conclusions:

1. Despite various theories trying to explain the origin of PD, the mechanisms are remained unknown. Our findings revealing the malfunction of amine concentration in similar organelles to secretory vesicles of dopaminergic neurons, give direct support to the dopaminergic theory and would clarify the origin of Parkinson's disease.

2. We have found a drastic decrease in 5-HT content of platelets from PD patients, along with a significant alteration in the 5-HT-uptake process in these patients, leading to the possibility of using this analytical method for the preclinical diagnosis of PD, probably decades prior the appearance of the first symptoms.

3. We have established our threshold limits for basal 5-HT concentration in platelets in 2.08 ± 0.09nmol|mg protein|$^{-1}$, for control group and 0.30 ± 0.03nmol|mg protein|$^{-1}$, for PD patients (mean ± SEM).

4. Our results clearly show a drastic reduction in both basal and serotonin uptake by human platelets from PD patients. Especially in basal content no overlapping data were between controls and patients being the first analytical tool with 100% reliability.

WHAT HAVE I LEARNT BY DOING THE FINAL GRADE PROJECT?

Thanks to this Final Grade Project, I have had the opportunity of spending time at one of the laboratories in our faculty, and above all, of learning how to work with many different instruments there. During the Grade of Medicine, we hardly did not spend any time at the laboratory, so almost everything was new for me.

Thus, I have learnt, from how to use pipettes and plates or prepare solutions, to how to analyse data with a program I had to install on my laptop. Along with how the different devices work and what they are used for, like the centrifuge, HPLC-ED, spectrophotometer, plate-reader…

It is really a pity that we devote at least 6 years studying medicine and we do not have any idea, even of the simplest things, about working at a laboratory. This is the main reason why I have chosen my project to be at the laboratory and I could not be more grateful.

Therefore, I am entirely thankful to my tutors (Dr Ricardo Borges and Dr Pablo Montenegro) for their great patience, will and wisdom, and for their teaching labour.

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APPENDIX 01

Nº Ficha:.....

FICHA CLÍNICA

ANTECEDENTES PERSONALES

DNI/NIF (tres últimas cifras): XXXXXX..... Letra:.....

Fecha de nacimiento (aaaa/mm/dd):...../...../.....

Edad:.....

Sexo (V/M):.....

ANTECEDENTES CLÍNICOS

Enfermedades conocidas:.....

.....

.....

.....

Medicamentos y fármacos que toma actualmente (excepto anticonceptivos):.....

.....

.....

.....

Antecedentes de enfermedades neurológicas (Parkinson, epilepsia, etc.):.....

.....

.....

.....

Síntomas premotores (Estreñimiento, problemas de olfato, sistemas depresivos, etc.):

Si ☐

No ☐

¿Desea ser contactado?

Si ☐ Número de teléfono:.....

No ☐

ANEXO VI. MODELO DE CONSENTIMIENTO INFORMADO POR ESCRITO

Por favor conteste las siguientes preguntas:
(Deberá señalar con un círculo sí o no)

¿Ha leído la hoja de información al paciente? SI NO
(Por favor, guárdese una copia)
¿Ha recibido contestaciones satisfactorias a todas sus preguntas? SI NO
¿Ha recibido suficiente información sobre el estudio? SI NO
¿Quién le ha explicado el ensayo clínico?:
Dr./Dra._____

¿Ha comprendido que se puede retirar del estudio…?:
- en cualquier momento SI NO
- sin dar razones de su retirada SI NO
- sin afectar a su futura atención médica SI NO

Los datos clínicos relativos a su participación en el ensayo, serán revisados y/o auditados por personal autorizado por el promotor y posiblemente por inspectores de las Autoridades Sanitarias, tanto españolas como europeas.

Todos los datos personales serán tratados en estricta confidencialidad.

¿Da usted su permiso para que estas personas tengan acceso a su historia clínica? SI NO
¿Ha tenido tiempo suficiente para tomar una decisión? SI NO
¿Está de acuerdo en tomar parte voluntariamente en el estudio? SI NO

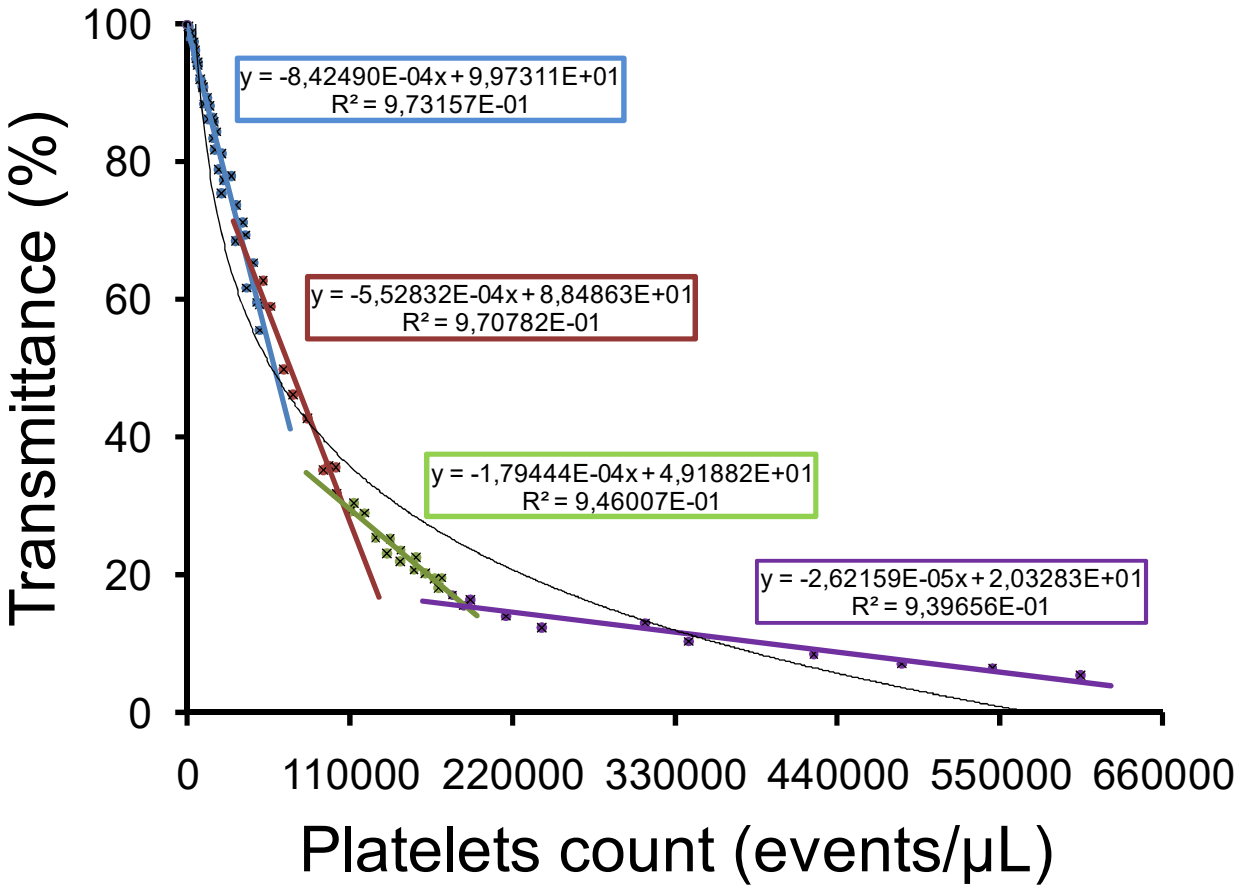
Usted sólo podrá participar en el ensayo si todas las respuestas son afirmativas.

Presto libremente mi conformidad para participar en el estudio.

Firma del paciente Fecha_____
Nombre y Apellidos (mayúsculas, letra clara)
_____

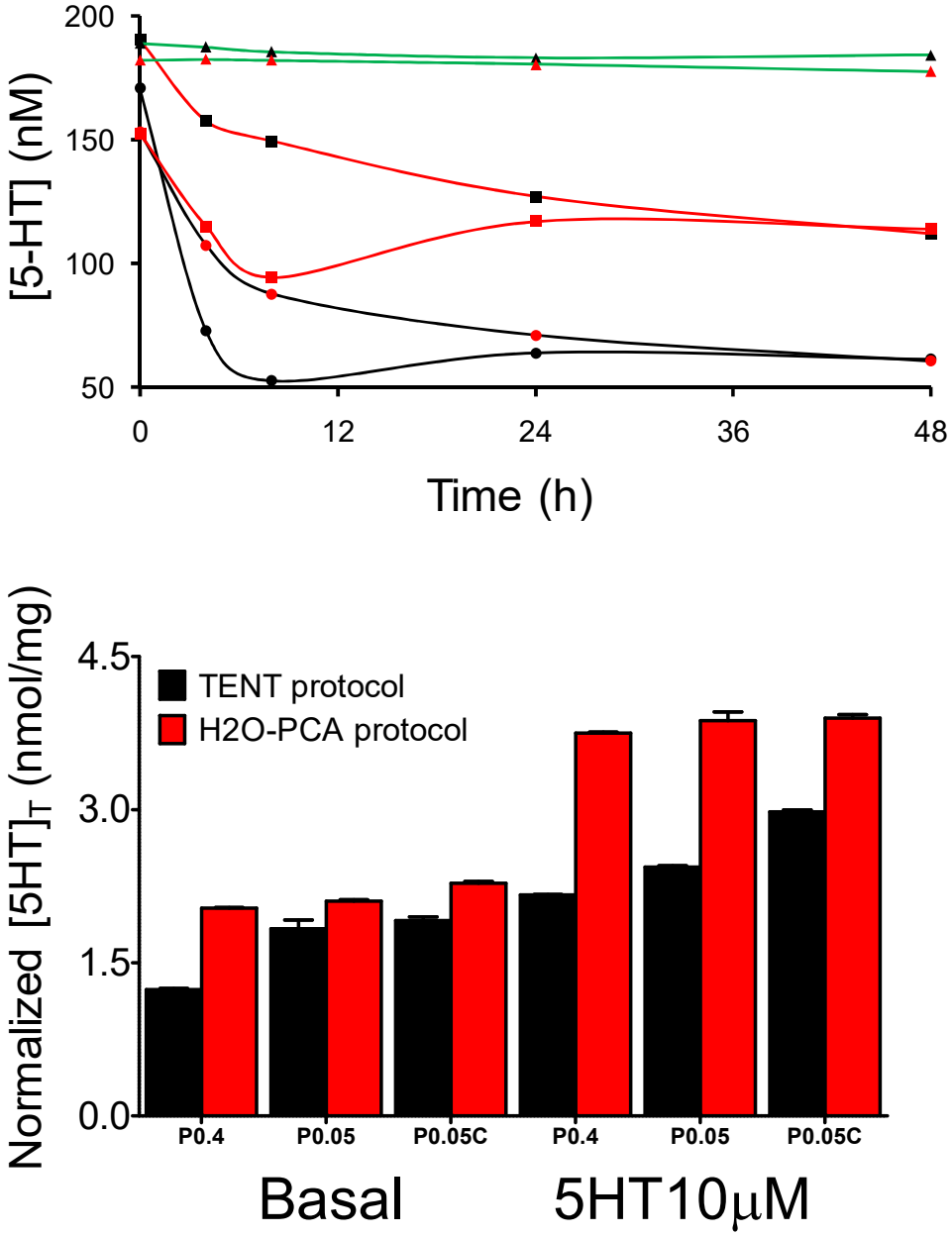
Appendix 01. Clinical form used in the present study for all human platelet random donors of Servicio de Neurología del Hospital Universitario de Canarias (HUC), Centro Neurológico Dr Antonio Alayón and Facultad de Ciencias de la Salud (Sección de Medicina) de la Universidad de la Laguna (ULL).

APPENDIX 02



Appendix 02. Turbidimetric correlation of the number of platelets for counting. It is represented the data of light transmittance versus exact count by flow cytometry (represented as number of events per microliter). Each of the equations represents an optimal linear range of platelet count, usually only using the corresponding blue trace.

APPENDIX 03



Appendix 03. Serotonin (5-HT) stability assays. In the upper graph is observed the stability of 200nM 5-HT against time in different TENT (50mM Tris-HCl, 5mM EDTA, 150mM NaCl, 1% TritonX, pH7.4 NaOH) platelets resuspended media: 0.4N PCA (black), 0.05N PCA (red) and 0.05N PCA supplemented with 300µM EDTA and 300µM L-Cysteine (green). The symbols of the tracings in turn represent different types of vial insert where the 5-HT- acid solutions were stored. In the lower graph are shown measures of basal [5-HT] as well as 5-HT10uM uptake with various media and protocol.