MONALISA: MOlecular NAnotechnology for LIfe Science Applications

NANOTEC-CNR (LECCE)

G. SCOLES, & D. COGLITORE
FROM MY PROBLEM TO THE DIAGNOSTICS OF RARE NEURO-DEGENERATIVE DISEASES
SCIENTIFIC EVENING OF OCTOBER 16, 2019

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Solutions (even cute 😊) in search of a problem to solve or PROBLEMS (even bad) in search of a solution that we want and can provide?

Metastatic cancer and Liquid biopsies

This has been the work of the last years. Now it is time to **Understand degenerative diseases**

(maybe it is too late for me, but I am trying anyhow!)

**BUT FIRST LET ME NOTE THAT:** IT IS VERY IMPORTANT TO GET THE CORRECT DIAGNOSIS FOR ANY DISEASE! my problem started in 2006 (micrografia) and for reasons that will become clearer later I lost several years
THERE I WAS in JANUARY ‘18 JUST BEFORE GOING TO PHILADELPHIA

I WAS STILL CONVINCED THAT I HAD A RARE FORM OF PD (Parkinsonism) WITH THE FOLLOWING SYMPTOMS:

1) COMPLETE LACK OF RESPONSE TO LEVODOPA THERAPY

2) NO TREMORS WHATSOEVER.

3) ALMOST CONSTANT FREEZING OF THE GAIT

4 TOTAL Micrographia (FIST SYMPTOM APPEARING)

5) RAPIDLY PROGRESSIVE BLEPHAROSPASM APPEARED AFTER A FEW YEARS SINCE DIAGNOSIS

BUT already 5 or 6 years before I HAD FOUND A 2002 paper that maintained that exactly these symptoms corresponded to a different illness that is: PURE AKINESIA WITH FREEZING OF THE GAIT (now a form of PROGRESSIVE SUPRANUCLEAR PALSY or PSP) Unfortunately this paper did not mention that the presence of TAU protein was an essential part of the diagnosis.
BUT I HAD ALREADY DISCUSSED WITH MY NEUROLOGIST IN 2008

A 2002 PAPER THAT DISCUSSED THE POSSIBLE EXISTANCE OF A PURE AKINESIA WITH GAIT FREEZING SYNDROME

HOWEVER NOTHING CAME OF IT MAINLY BECAUSE
1) THE DOCTOR HAD NEVER SEEN A PATIENT LIKE ME and
2) THE 2002 PAPER NEVER SPOKE OF THE TAUOPATHIC NATURE OF THE SYNDROME THAT WOULD HAVE ESTABLISHED ITS LINK WITH THE PROGRESSIVE SUPRANUCLEAR PALSY (PSP)

3) THIS LINK WAS ESTABLISHED LATER WITH A VERY CAREFUL CLINICAL POST MORTEM STUDY THAT USED THE LONDON QUEEN SQUARE BRAIN BANK
After having wasted some time, I started looking out of the BOX. But before we look into that we need to do a few preliminary things SUCH AS ASKING OURSELVES THE FOLLOWING QUESTION:

IS IT POSSIBLE TO ARRIVE AT A GOOD DIAGNOSIS BY CLINICAL OBSERVATION AND HOW DOES IT GET IT DONE?

POST VS PRE-MORTEM DIAGNOSTICS!
So how a good diagnose gets done at present?

The first condition is that the doctors that evaluate you MUST have seen a few patients like you (a dozen or so).

The second is that the doctor that examine you must be up to date with the literature.

The center for movement disorders at U PENN where dr. DAVID COUGHLIN USED TO WORK TILL A FEW MONTHS AGO with a fresh PhD in PSP (12 cases) (NOW @ U. of C. San DIEGO) is one of the 2 places in the USA (THE OTHER BEING THE Mayo CLINIC IN Arizona (40 cases) where difficult cases can be found.
Pure akinesia with gait freezing a third phenotype of PSP

Abstract

The clinical syndrome of pure akinesia has most often been associated with progressive supranuclear palsy (PSP) and is characterized by difficulty initiating gait and "freezing" during walking, writing and speaking. Similar syndromes have been described under the rubrics of primary progressive freezing gait and primary gait ignition failure. We investigated the specificity of the clinical syndrome of pure akinesia with gait freezing (PAGF) for PSP-tau pathology. INVESTIGATING the BRAINS at the Queen’s Square Brain Bank or QSBB POST-MORTEM!!
THE STUDY USING THE QSBB

Among 749 patients archived at the QSBB, only 7 fulfilled proposed diagnostic criteria of: gradual onset of freezing of gait or speech; absent limb rigidity and tremor; no sustained response to levodopa; and no dementia or ophthalmoplegia in the first 5 years of disease. In these cases detailed pathological examination was performed. PSP was the pathological diagnosis in six patients, and alpha synuclein(PD) in the seventh. Since the prevalence of PD is approx. 1% and PSP-Richardson. 1% of that (i.e. 1 part in 10,000) 1% of that gives a 1 case per 1/2M population.
In these six with PSP there were no additional features of coexistent vascular or PD (alphasyn) and the median PSP-tau score was 3, reflecting relative mild tau load.

The clinical syndrome of PAGF appears to have a high specificity for PSP-tau pathology. This relatively uncommon presentation of PSP-TAU pathology has less severe TAU accumulation than in the more common, "classic" PSP clinical phenotype: Richardson's disease which kills you in 8 years +/- 6 months a very narrow distribution!

And this adds another diagnostic criterium because for instance if I had RICHARSON syndrome I would be already dead (SEE THE NEUROLOGIST IN UDINE)

(c) 2007 Movement Disorder Society
Uses of TAU & state of the art

Comparative studies using autopsy-confirmed samples suggest that CSF total-tau (t-tau) and Aβ(1-42) levels can accurately distinguish FTLD from AD, with a high t-tau to Aβ(1-42) ratio diagnostic of AD.

The limit of detection of the assay, which requires 30 μL of plasma, is 0.02 pg/mL (or 0.02 ng/L) (or ~0.5 fM concentration) [2], which is more than 1,000-fold more sensitive than conventional immunoassays. [Indeed elisa tests are said to have ~ 1 pM conc. detectability]

BEST NOT TO LET THE PROTEIN OUT OF THE CELLS
Because this level of sensitivity is unheard about, we need to look carefully into it!
Enter Daniela Cesselli and her study of Niemann Pick disease!

DURING HER TENURE IN MY ERC GROUP WHILE I WAS GOING IN AND OUT OF HOSPITALS IGNORING THE GAIT FREEZING (THREE SHOULDER OPERATIONS AND ONE SHOULDER PROTESIS INSTALLED) SHE DECIDED TO USE STEM CELLS TO ARRIVE AT A MOLECULAR MODEL OF A GENETIC DISEASE: NIEMANN PICK!
AUTOSOMAL RECESSIVE INHERITANCE NEUROVISCERAL LYSOSOMAL STORAGE DISORDER, CAUSED BY THE ABNORMAL FUNCTION OF NPC1 OR NPC2 PROTEIN.

THE DEFICIENCY OF EITHER OF THEM LEADS TO THE ACCUMULATION OF THE ENDOCYTOSED UNESTERIFIED CHOLESTEROL, GANGLIOSIDES AND OTHER LIPIDS WITHIN THE LYSOSOME/LATE ENDOSONE (LE) COMPARTMENT.

NPC1 GENE IS MUTATED IN 95% OF THE FAMILIES, NPC2 IS INVOLVED IN RARE FAMILIES.
**Niemann Pick-C disease: open questions**

**Despite the progress in characterizing the biochemical and genetic defects in NPC disease, the mechanisms underlying the pathophysiology of this disorder are not clear and the currently available therapeutic interventions are limited:**

**NP-C patient management is often symptomatic**

**Translation of data obtained from animal models to human patients is not simple**

**Limited availability of human neuronal models of the disease:**
- Fibroblast accumulate cholesterol only
- Neurons accumulate cholesterol and gangliosides GM2 and GM3
FROZEN FIBROBLAST CULTURE: 
n=3 NP-C patients 
n=3 Healthy Controls

FRESH SKIN BIOPSY

CULTURE IN A MEDIUM SELECTIVE FOR THE GROWTH OF HUMAN MULTIPOTENT ADULT STEM CELLS (hMASC)

IN VITRO INDUCTION OF NEURAL DIFFERENTIATION
Neural differentiation

CTRL

NP-C
AFM characterization of differentiated cells

Dr. Anita PALMA

CTRL

MAP-2 DAPI

HEIGHT IMAGE

AMPLITUDE IMAGE

NP-C

MAP-2 DAPI

HEIGHT IMAGE

AMPLITUDE IMAGE
From Walkley et al., BBA 2004 48/62

**CTRL**

**NP-C**

Meganeurite

Ectopic dendritogenesis
Conclusions

It is possible to develop from NP-C patients a neural in vitro model resembling some crucial NP-C in vivo alterations:

1. Abnormal lipid accumulation (cholesterol and GM2 ganglioside)
2. Morphological changes (meganeurite, ectopic dendritogenesis)
This “PERSONALIZED” in vitro model can be used to:

1. understand the molecular basis of NPC neurodegeneration;

2. evaluate the impact of different mutations on the pathological phenotype;

3. perform drug screening on cells obtained from NPC patients presenting different genotypes;

4. generate human neuronal models of other neurodegenerative diseases
Main advantages:

1. It was developed directly from patient’s cells and therefore it would be useful to analyze the effect of specific NPC1 mutations within the context of the patient genetic background;

2. It was obtained through the differentiation of cells obtained from accessible sources;

3. It did not involve the forced expression of transgenes in target cells, thus avoiding confounding results due to the reprogramming process (iPS).
PROJECT: SCOLES
Uses of TAU & state of the art

Comparative studies using autopsy-confirmed sample suggest that CSF total-tau (t-tau) and Aβ(1-42) levels can accurately distinguish FTLD from AD, with a high t-tau to Aβ(1-42) ratio diagnostic of AD. The limit of detection of the assay, which requires 30 μL of plasma, is 0.02 pg/mL (or 0.02 ng / L) (or ~0.5 fM concentration) [2], which is more than 1,000-fold more sensitive than conventional immunoassays. [Indeed Elisa tests are said to have ~ 1 pM conc. detectability]

Because this level of sensitivity is unheard about, we need to look very carefully into it!
BY INTUITION I DECIDED TO LOOK INTO USING THE SAME SYSTEM FOR SPORADIC DISEASES AND SHE WENT ALONG WITH ME AND TRIED! ONLY DIFFERENCE WAS THAT WE STARTED FROM ADIPOSE TISSUE INSTEAD THAN FROM SKIN!
FRESH SKIN BIOPSY

SUB-CULTURE IN A MEDIUM SELECTIVE FOR THE GROWTH OF human MULTIPOTENT ADULT STEM CELLS (hMASC)

ADIPOSE TISSUE (LIPOSUCTION)

IN VITRO INDUCTION OF NEURAL DIFFERENTIATION

CHARACTERIZATION:
• FACS
• IF
• CLONOGENICITY
• MULTIPOTENCY

STORAGE OF FROZEN VIALS
THE VERY ITALIAN STORY OF ANNA BERTOLINI

A TECHNICIAN HAPPY TO BE THAT................................
Seconda prova di differenziamento

DONATORI SANI: H-SLIM 121 (75 ANNI); H-SLIM 130 (50 ANNI)

PAZIENTE: GS (84 ANNI)
NEURAL DIFFERENTIATION PROTOCOL

STEM CELL

N1
24h
DMEM HG 10%FBS

N2
5 days
1% B27
10ng/mL EGF
20ng/mL bFGF

N3
48h
DMEM HG
5µg/mL insulin
200µM indometacin
0,5mM IBMX
TAU: H-SLIM 121
TAU: H-SLIM 130
Nota 1: presenza di cellule molto più grandi (ingrandimento invariato)
Nota 2: presenza di cellule con prolungamenti così lunghi da staccarsi e risultare deformati (credo durante le varie procedure di colorazione). Forse la densità in questi vetrini era molto più bassa e ha spinto alla formazione di prolungamenti.
Nota 2: altro esempio di cellule con lunghi prolungamenti
MN1000: H-SLIM 121

L’anticorpo ha un pattern diverso rispetto a TAU:
1. Marca le membrane, soprattutto nei prolungamenti e nelle estremità;
2. Si marcano formazioni vescicolari
3. Nel caso 121, ci sono cellule molto positive altre di meno.
L’anticorpo ha un pattern diverso rispetto a TAU:
1. Marca le membrane, soprattutto nei prolungamenti e nelle estremità;
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3. Nel caso 121, ci sono cellule molto positive altre di meno.
L’anticorpo ha un pattern diverso rispetto a TAU:
1. Marca le membrane, soprattutto nei prolungamenti e nelle estremità;
2. Si marcano formazioni vescicolari
3. Nel caso 121, ci sono cellule molto positive altre di meno.
AT8: H-SLIM 121

Nota: si nota solo una debole positività nucleare puntinata
AT8:
H-SLIM 130

Nota: si nota solo una debole positività nucleare puntinata
AT8: GS

Nota: il pattern è diverso. C’è una debole positività del citoplasma e la presenza di alcuni «accumuli»: vedi MN1000/GS2
AT8: GS
ALL OF MY MARVELLOUS TEAM FOR HELP

ACKNOWLEDGEMENTS

«MENTORS»: A. De Marco (N.Go) A. Laio (Sissa) S. Gustincich (Sissa) L. Casalis (Elettra) M. Lazzarino (IOM-CNR) S. Fortuna & M. Soler (N. Gorica & Sissa)

AND MY MOST PATIENT WIFE GIOK-LAN
In my opinion now is the time to start looking for a cure!

NOT BEFORE WE HAVE “COMPLETED” THE CURRENT REVIEW OF THE DIAGNOSTICS METHODS.

FOR INSTANCE LOOKING INTO THE METHOD OF EVALUATING THE DNA DAMAGE IN LEUCOCITICES OBTAINED FROM THE PERIPHERAL BLOOD PLASMA (see paper by VEA & Porcellini) VEA=

V. ENRICO AVVEDIMENTO (Mr. Epigenetics)
CESSELLI’S WORK ON NIEMANN PICK DISEASE

Genetic diseases are relatively easy

WP1: Sviluppo di un modello in vitro di taupatia a partire dalle cellule staminali multipotenti dell’adulto (MASC) isolate da biopsie cutanee. Leader: UniUD.

An application will be described to the diagnostics of neurodegenerative diseases choosing the area where the methods would be more beneficial (i.e. the area of rare diseases). We will consider a class of TAUopathies, known as Progressive Supranuclear Palsy (PSP), both in their main phenotype and in the rare PAGF phenotype i.e./ Pure Akinesia with Gait Freezing that has an "abundance" of 1 over 0.5 million individuals. Our approach involves the production of pluripotent stem cells from the adipose tissue of a well, clinically diagnosed, individual and their subsequent differentiation to neurons. If the latter result to be diseased then the road to the cure is possible and well planned. The main difficulty is in the complexity of the brain that sees, for example, the localization of the disease in the brain stem. The method has been, however, already proven to be effective in genetic diseases but our interest involves the more difficult case of sporadic diseases.
Our goal is to establish the level of production of protein TAU and compare it to the cells of a healthy individual from the adipose issue of which the neurons will be produced following a similar protocol. Two complementary approaches will be followed: (i) if the cellular dilution factor (diseased neurons over the total number of neurons obtained) will not prove to be too limiting, we will be able to proceed directly for the molecule that may wash out the extra amount of TAU. Otherwise (ii), we will need to go to the second level looking to establish the presence of disease molecular fingerprint at the single cell level establishing, in such way, the heterogeneity of the disease. We plan to do that by means of nanopipettes that will have their interior coated with antibodies or nanobodies that recognize the TAU protein. When the nanopipette suck out some of the cell fluid the TAU will stay inside the nanochannel of the pipette changing its ionic conductivity.
ACKNOWLEDGEMENTS

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AND MY MOST PATIENT WIFE GIOK-LAN
AT8: H-SLIM 121

Nota: si nota solo una debole positività nucleare puntinata
Nota: il pattern è diverso. C’è una debole positività del citoplasma e la presenza di alcuni «accumuli»: vedi MN1000/GS2.
In summary:

1) I need to establish that my illness is a TAUopathy
   a) to confirm the diagnosis and b) to decide to ask to participate in a phase 3 study conducted at U PENN with an antibody that is supposed to clear your brain from TAU. But the majority of participants have PSP-Richardson (level of TAU in CSF 200 times that in blood for healthy and diseased persons.

c) even in the case that I confirm the presence of tau in my peripheral blood it is not at all sure that I ask to participate in the study unless they show me that the class of patients with PSP-PAFG has been considered separately and the results have been found satisfactory including side effects.