Original article

Olfactory neuroepithelium alterations and cognitive correlates in schizophrenia

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ABSTRACT

Background: Few studies have investigated alterations of olfactory neuroepithelium (ONE) as a biomarker of schizophrenia, and none its association with cognitive functioning.
Method: Fresh ONE cells from twelve patients with schizophrenia and thirteen healthy controls were collected by nasal brushing, cultured in proper media and passed twelve times. Markers of cell proliferation (BrdU incorporation, Cyclin-D1 and p21 protein level) were quantified. Cognitive function was measured using Brief Neuropsychological Examination-2. Primary outcome: proliferation of ONE cells from schizophrenic patients at passage 3. Secondary outcome: association between alteration of cell proliferation and cognitive function.
Results: Fresh ONE cells from patients showed a faster cell proliferation than those from healthy controls at passage 3. An opposite trend was observed at passage 9, ONE cells of patients with schizophrenia showing slower cell proliferation as compared to healthy controls. In schizophrenia, overall cognitive function (Spearman's rho -0.657, p < 0.01), verbal memory – immediate recall, with interference at 10 s and 30 s (Spearman's rho from -0.676 to 0.697, all p < 0.01) were inversely associated with cell proliferation at passage 3.
Conclusion: Fresh ONE cells collected by nasal brushing might eventually represent a tool for diagnosing schizophrenia based upon markers of cell proliferation, which can be easily implemented as single-layer culture. Cell proliferation at passage 3 can be regarded as a promising proxy of cognitive functioning in schizophrenia. Future studies should replicate these findings, and may assess whether ONE alterations are there before onset of psychosis, serving as an early sign in patients with at risk mental state.

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1. Introduction

Schizophrenia is a chronic mental disorder, associated with a life-expectancy reduction of about 14.5 years compared with the general population. [1] Schizophrenia is associated with severe psychosocial functioning impairment, which is strongly connected, as shown in a recent network analysis on a large set of patients, with cognitive symptoms [2].

Several studies have demonstrated that aberrant genes, ethnicity, early neural hazards are associated with neurodevelopmental alterations, [3–5] and that together with environmental risk factors in adolescence and early adulthood [4–6] play a role in development of schizophrenia. Despite such convincing evidence on risk factors for psychosis, evidence on biomarkers remains limited [4]. Among several putative biomarkers of schizophrenia, few studies have investigated proxy of neurodevelopmental alterations, such as alterations of olfactory neuroepithelium (ONE).

Postulated about three decades ago, the neurodevelopmental hypothesis for the etiology of schizophrenia has now become the leading and most widely accepted model. [6,7] Neural development is a complex process in which specific neural progenitor cells (NPCs) proliferate, differentiate into numerous cell types, migrate to their final positions, and ultimately form integrated circuitries [8,9]. Neurogenesis is generally followed by gliogenesis, with the same progenitor domains switching the differentiation program to oligodendrocyte or astrocyte production [10]. Interference with cell proliferation has been identified as a putative common denominator for a number of environmental and genetic disturbances of embryonic development [11]. Given the complexity and the "intertrimming compartmentalized" nature of neurodevelopment since the early proliferative stages, it appears clear that a major requirement for this process to succeed is the coordination of cell cycle regulation and cell fate determination [12,13].

The majority of neuroepithelial precursors in mammalian adult cells are maintained in a quiescent state (G0 phase); [14,15] quiescent cells can re-enter the cell cycle in G1 phase when exposed to appropriate mitogenic stimuli [16]. Transitions through the cell cycle are driven by cyclins and cyclin-dependent kinases (CDKs) [17]. Cyclins are the regulatory subunits of CDK and are degraded or synthesized during cell cycle; CDKs activity is strictly regulated by the levels of cyclin partners, phosphorylation status and the abundance of CDK inhibitory proteins, such as p21 [17]. Cyclins Ds, including D1, are the first cyclins sensing the mitogenic signals and therefore, as growth factor sensors, they activate CDK4 and CDK6 in G1 phase. Cyclin expression, accumulation and degradation, as well as assembly and activation of CDK4/CDK6 are mainly governed by growth factor stimulation. The CDK inhibitor p21 is a well-known inhibitor of cell cycle and can arrest the cell cycle progression in G1/S and G2/M transitions by inhibiting CDK4,6/cyclin-D and CDK2/cyclin-E, respectively [18]. Given the aforementioned, Cyclin D1 and p21 could represent a viable molecular marker of cellular transition from quiescent to proliferative phase.

Notwithstanding the differences in neurogenic processes occurring in embryos with respect to those occurring in the staminal compartment of the adult [19], a promising model to study cell proliferation is the isolation and culture of tissue specimens from the human ONE [20]. Cells from ONE are particularly suited as models of brain diseases, since they have a common ectodermal embryonic origin with central nervous system neurons, they maintain the capacity to proliferate and differentiate into neurons without genetic reprogramming, are easily and safely accessible in humans, and demonstrate both dynamic changes (state) and persistent signatures (trait) directly associated with disease at the molecular level [20–23].

Few previous studies investigated ONE cells proliferation in schizophrenia. One group in Australia showed in two studies [22–24] an increased number of mitotic cell in patients with schizophrenia compared with control. These findings could reflect higher rates of cell proliferation in schizophrenia. The same group in a study published more recently showed an increase of cell proliferation with higher expression levels of cyclins D1, E, and A2 in schizophrenia compared with control cells sampled by means of nasal biopsy [21]. However, only one study has sampled ONE cells with nasal brushing, and no study investigated any correlation between ONE single-layer cells cultures and clinical variables. Identifying a biomarker of schizophrenia eventually correlating with cognitive impairment or other predictors of poor outcome might be useful to detect at early stages those cases which might be benefit from pro-cognitive treatments in the future.

The main aim of this study is to test whether single-layer cultures of ONE fresh cells sampled with nasal brushing in patients with schizophrenia confirm previous findings on neurosphere of ONE cells collected with nasal biopsy, and to advance the knowledge by investigating the cell proliferation trend over passages, and whether ONE cells alterations are associated with clinical variables, cognition in particular.

2. Methods

2.1. Inclusion and exclusion criteria

A total of 12 patients and 13 healthy controls were initially recruited among those admitted as inpatients at the Psychiatry Inpatients Unit, Padua University Hospital, in a period ranging from september 2017 to november 2018.

Patients's inclusion criteria were age between 18 and 65 years old and a current diagnosis of Schizophrenia according to DSM-5, with previous multiple episodes, clinically stabilized after admission for acute symptoms relapse, and right before discharge. [25] Exclusion criteria were a diagnosis of substance related and addictive disorder according to DSM-5 [25] criteria in the last 12 months, comorbidity with other psychiatric disorders, history of neurological disease (including dementia) or head trauma with a loss of consciousness of at least 5 min.

Healthy controls' inclusion criteria were not having a psychiatric condition according to DSM-5, [25] nor any major neurological or medical illness.

All participants provided written informed consent prior to their involvement in the study, in accordance with the Helsinki ethical principles for medical research.

2.2. Materials

Culture media and fetal bovine serum were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA, USA). Anti-nestin antibody, monoclonal anti-βIII-tubulin antibody and anti-glial fibrillary acidic protein (GFAP) antibody were from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Alexa fluor 488-conjugated goat anti mouse antibody was from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA). Cell Proliferation ELISA, BrdU (chemiluminescent) was purchased from Roche Diagnostics (Manheim, Germany). Primocin was from Invivogen (San Diego, CA, USA).

2.3. Isolation and culture of olfactory neuro-epithelium cells

Olfactory neuroepithelial fresh cells were obtained from the nasal middle turbinate with a dedicated brusher as previously described. [26] Briefly, an ear-nose-throat MD specialist performed a nasal endoscopy with a 0° and/or 30° rigid endoscope in the more accessible of the two nasal cavities, and scraped the ONE cells from the surface of middle turbinate with the dedicated brush, avoiding contact with surrounding tissues. Disinfection with clorexidine 0.5% in the nasal vestibule was performed when necessary (nasal vestibulitis, presence of significant nasal discharge).

The specimen was then harvested in Dulbecco's modified Eagle and F-12 media (DMEM/F-12) supplemented with 10% fetal bovine serum, 4 mM l-glutamine, and Primocin™. Importantly, the collection of the specimens was exclusively carried out in spring and summer to avoid seasonal infections that would have required the supplementation of the media with antibiotics that showed to be detrimental to the growth and survival of ONE cells in pilot cultures. To dissociate cellular aggregates, ONE cells were then passed through a micropipette tip 20 times and eventually cultured in DMEM/F-12 medium supplemented as above.

2.4. Immunofluorescence staining on ONE cell culture

4×10^4 ONE fresh cells were seeded on a coverslip and let adhere for 24 h; cells were fixed with 4% paraformaldehyde and then washed with PBS 1X supplemented with 0.5% Triton X-100; following 10 min incubation with UltraVision protein block (ThermoScientific), to avoid aspecific staining, cells were incubated with mouse anti-βIII-tubulin, a marker for neuralprogenitors and developing neurons, for 1 h at room temperature. Cells were then washed with PBS 1X + 0,5% Tween 20 and then incubated for 45 min with the appropriate secondary Alexa fluor 488-conjugated antibody (1:500, ThermoFisher). Cells were then rinsed and mounted with Vectashield with DAPI (Vector Labs) to counterstain nuclei. Micrographs were taken using an Eclipse E800 microscope equipped with a DS-U1 cooled digital camera and analyzed using the LuciaG software (all from Nikon).

2.5. Cell proliferation assay (BrDU incorporation)

Proliferation assays were performed by using Roche Cell Proliferation ELISA, BrdU (chemiluminescent). Briefly, 2.5×10^3 fresh cells were seeded on 96-well flat bottom microplates and incubated in DMEM/F-12 medium alone for 24 h to stop cell growth. Cells were preincubated at time points as indicated in the Results. ONE cells were labeled with 10 μM BrdU 10 h before each single incubation time point. The following treatment was carried out according to the manufacturer's guidelines. The microplates were then washed three times with washing solution and 100 μl substrate solution was added and incubated for 5 min. Absorbance of the samples was measured using a luminometer.

2.6. Western blotting (Cyclin-D1, p21)

Total cell lysates were run in 10% SDS-PAGE and transferred onto nitrocellulose membranes. After 1 h of treatment with 3% bovine serum albumine at room temperature, membranes were incubated with the appropriate antibodies overnight. Immunodetection was carried out with the ECL Western Blotting Substrate on the Kodak Image Station 4000 mm Pro Digital System (Eastman Kodak, Rochester, NY, USA). Membranes, when required, were reprobed with other primary antibodies after stripping with 0.1 M glycine (pH 2.5), 0.5 M NaCl, 0.1% Tween 20, 1% β-mercaptoethanol and 0.1% NaN3 for 2 x 10 min.

2.7. Cognitive assessment

Cognitive function has been assessed with Brief Neuropsychological Examination-2 [27] (ENB-2), administered by two trained clinical psychologists, in a time interval ranging from 2 days before and 2 days after the nasal endoscopic procedure.

ENB-2 includes 16 subtests (Digit span, Immediate and Delayed recall prose memory, Interference memory at 10 and 30 s, a Trial making test parts A and B, Token test, Word phonemic fluency test, Abstract reasoning test, Cognitive estimation test, Test of overlapping figure, Spontaneous drawing, Copy drawing, Clock drawing, and Ideative and ideomotor praxis test).

ENB-2 evaluates several cognitive domains, namely attention, executive functioning, perception, praxis abilities and comprehension, plus an overall cognitive score. every single test in the cognitive battery explores more than one function, encompassing different cognitive domains as defined by DSM-5. [28] For example, trail making tests evaluate selective, divided and alternate attention and working memory, alongside with visuospatial research capacity and psychomotor speed. The battery has proven to show good psychometric characteristics, revealing good differential validity in discriminating normative and clinical groups and sufficient test-retest reliability [29].

2.8. Primary and secondary outcomes

Primary outcome of the present work is the difference in ONE fresh cells proliferation between patients with schizophrenia and healthy controls as soon as passage 3.

Secondary outcome is to test the correlation of ONE fresh cell alterations with cognitive function within patients with schizophrenia.

Other outcomes will be fresh cell proliferation alterations at passages 6, 9, 12 and safety of ONE cells collection procedure (nasal brushing).

2.9. Statistical analysis

For statistical analysis we used SPSS 20 [30] and Graphpad Prism 8 [31].

We used demeaned BrdU incorporation levels and of Cyclin D1 and p21 protein levels, as they were determined for each subject at least twice per point. Homogeneity of the two population has been tested using chi-squared test for the principal demographic and clinical variables collected. Kolgomorov-Smirnov test was used to test the normality of data distribution and to choose the more appropriate statistical approach for group comparison: parametric tests were used where data were normally distributed (Student's t-test), non-parametric tests for non-normally distributed data (Mann-Whitney U). For correlation analysis we calculated Spearman's rho correlation coefficient.

Statistical significance threshold was set at $<.05$.

3. Results

3.1. Population characteristics

Characteristics of included patients with schizophrenia and healthy control are reported at individual level in Table 1. Twelve patients and thirteen healthy controls participated to the study. Patients' and controls' mean age did not significantly differ [41.42 (15.19) vs 33.08 (12.69), p = 0.174], male gender was equally over-represented in the two samples (ten out of twelve vs ten out of thirteen, p = 0.689), and smokers rates did not differ between the two groups (five out of twelve vs six out of thirteen, p = 0.821]. Healthy controls received higher education than patients with schizophrenia [19.75 (2.93) years vs 12.25 (3.39), p < 0.001]. All patients were admitted at least once [mean admissions 2.42 (1.5)], all patients were treated with antipsychotics, and all except three patients were also taking benzodiazepines. All patients had been admitted for relapse of positive symptoms, namely paranoid delusions.

3.2. Primary outcome: fresh cell proliferation differences at passage 3

An image of ONE fresh cell cultures in healthy controls and patients with schizophrenia is shown in Fig. 1. Detailed results of cell proliferation measures at passage 3 are reported in Table 2 and 3, and shown in Fig. 2 with BrdU incorporation (analyzed by BrdU incorporation at discrete time points for 48 h), and Western blot analysis with antibodies against cyclin D and p21 as markers of the cell cycle at discrete time points for 24 h as well as βIII-tubulin as a loading control.

Fresh cell proliferation was higher at passage 3 in patients with schizophrenia than healthy control, as consistently shown by different markers, namely higher BrdU incorporation rIu/s (standard deviation) [schizophrenia 12.08 (0.21), healthy controls 7.99 (0.15), p < 0.001], higher Cyclin-D1 protein levels [schizophrenia 3391.79 (224.91), healthy controls 1656.48 (43.49), p < 0.001], and lower p21protein levels [schizophrenia 2006,77 (46,48), healthy controls 2717,67 (21,09), p < 0.001].

Table 1
Characteristics of included sample.

ID	Age	Education	Gender	Smoking	Admissions	Antipsychotic	CPZ 100 mg	Other medications
Patients								
1	28	11	M	Y	1	Aripiprazole	833	Delorazepam
2	41	13	F	N	4	Aripiprazole	833	
3	24	13	M	N	1	Aripiprazole	556	Valproic acid, lorazepam
4	43	8	M	Y	5	Risperidone	375	delorazepam
5	51	18	M	N	1	Risperidone	500	Diazepam, lispro insulin, glargine insulin, foline, ferrose gluconate, ramipril, hydrochlorothiazide
6	46	13	F	Y	4	Paliperidone, amisupride	950	Acetil-salicidic acid, diazepam,
7	64	8	M	Y	1	Perfenazine, quetiapine, zuclopenthixol	841	delorazepam
8	36	18	M	N	2	Amisulpride, quetiapine, aripiprazole	1100	lorazepam
9	65	8	M	N	2	Aripiprazole, clozapine,	437	biperidene, levothyroxine, mesalazine, linaclotide
10	19	11	M	N	1	clozapine, aripiprazole	577	Valproic acid, levothyroxine
11	27	13	M	N	3	clozapine, risperidone	1500	litio retard,lorazepam
12	53	13	M	Y	4	Clozapine, Haloperidol	937	diazepam,atorvastatin, doxazosin, ramipril, hydrochlorothiazide,
Overall [mean (SD); median (range)]	41.42 (15.19); 42 (19-65)	12.25 (3.39); 13 (8-18)	10 M, 2F	5 Y, 7 N	2.42 (1.50); 2 (1-5)		786.55 (381.19); 833 (375, 1500)	
Healthy controls								
1	31	21	M	N				
2	40	27	M	Y				
3	24	17	F	N				
4	27	19	M	Y				
5	25	18	M	Y				
6	55	24	M	Y				
7	62	18	M	N				
8	54	18	M	N				
9	28	19	M	N				
10	27	19	M	N				
11	25	18	F	N				
12	28	19	F	Y				
13	25	18	M	Y				
Overall [mean (SD); median (range)]	33.08 (12.69); 27.5 (24-62)	19.75 (2.93); 19 (17-27)	10 M, 3F	6 Y, 7 N	–	–	–	
Between patients and controls	0.174 Mann-Whithey U 48.5, p value = 0.174	<0.001 Mann-Whithey U 7, p value < 0.001	0.689 Chi-squared 0.16, p value = 0.689	0.821				

Legend. SD, standard deviation; * Mann-Whitney test for continuous variables, Chi-squared for categorial variables.

3.3. Secondary outcome: correlation of ONE fresh cells alterations at passage 3 and cognitive function in patients with schizophrenia

Correlations between all cognitive tests and cell proliferation as measured with BrdU incorporation at all fresh cell passages are reported in detail in Table 5. Brief neuropsychological examination (Spearman's rho -0.657, p < 0.01), verbal memory – immediate, recall, with interference at 10 s, with interference at 30 s ((Spearman's rho from -0.676 to 0.697, p < 0.01) were inversely associated with cell proliferation at passage 3.

3.4. Cell proliferation throughout subsequent cell passages

Results of fresh cell proliferation as measured with BrdUincorporation in patients and healthy controls from passage 3 to 12 are reported in detail in Table 2. Patients with schizophrenia showed a faster fresh cell proliferation than healthy controls [mean BrdU incorporation rIu/s (standard deviation)] at passage 3 [schizophrenia 12.08 (0.21), healthy controls 7.99 (0.15), p < 0.001] and passage 6 [schizophrenia 9.04 (0.06), healthy controls 8.96 (0.09), p = 0.035], with an inversion to lower cell proliferation at passage 9 [schizophrenia 6.19 (0.22), healthy controls 9.01 (0.8), p < 0.001] and passage 12 [schizophrenia 4.71 (0.05), healthy controls 9.03 (0.04), p < 0.001] (Fig. 3).

Results of cyclin-D1 protein levels in patients and healthy controls throughout cell passages 3–12 are reported in detail in Table 3. Cyclin-D protein levels were higher in schizophrenia than healthy controls at passage 3 [schizophrenia 3391.79 (224.91), healthy controls 1656.48 (43.49), p < 0.001], and passage 6 [schizophrenia 2001.67 (16.55), healthy controls 1841.09 (22.35), p < 0.001], with an inversion to lower protein levels at passage 9 [schizophrenia 1583.57 (32.52), healthy controls 1886.54 (30.54), p < 0.001] and passage 12 [schizophrenia 1129.62 (23.77), healthy controls 1933.91 (28.51), p < 0.001].

Results of p21 protein levels in patients and healthy controls throughout cell passages from passage 3 to 9 are reported in detail in Table 3. p21 was lower in schizophrenia than healthy controls in passage 3 [schizophrenia 2006,77 (46,48), healthy controls 2717,67 (21,09), p < 0.001], not significantly different at passage 6, with an
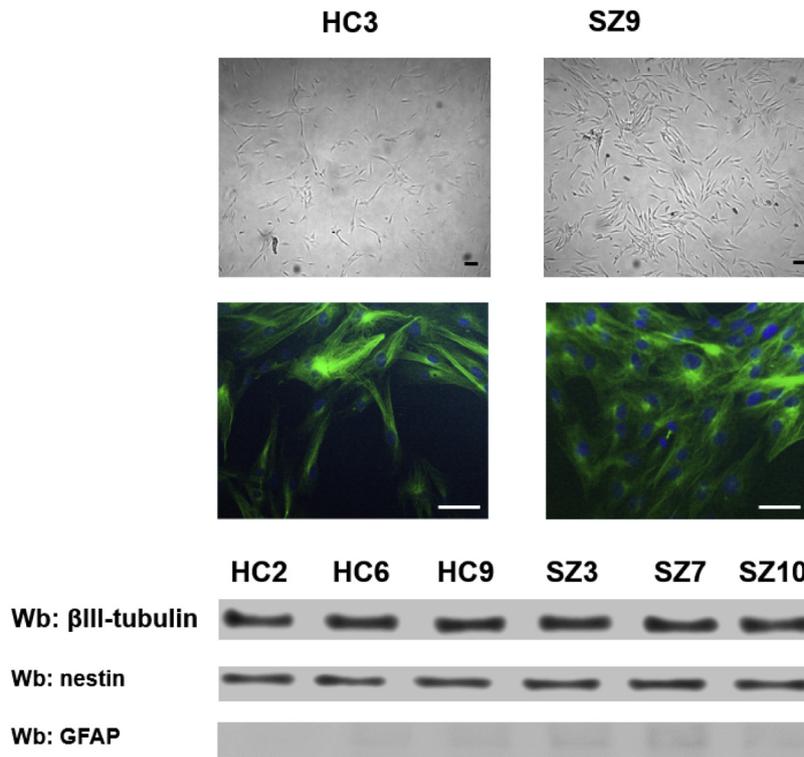
Fig. 1. Characterization of fresh ONE cells of healthy controls and patients with schizophrenia.
Legend. (A) ONE cells were examined after 24 h at passage 4 in culture medium by light microscopy. Bar =100 μm (upper images). ONE cells after 24 h at passage 4 in culture medium were fixed and stained with βIII-tubulin antibody and examined by immunofluorescence for immunofluorescence analysis (green). Diamidino-2-phenylindole dihydrochloride (DAPI) was used to stain the nuclei (blue). Bar =10 μm (lower images). (B) βIII-tubulin and nestin were detected by Western blot analysis as markers of neural stem cells. Anti-GFAP antibody was used to assess the proliferation of non-neuronal cells.

inversion to higher protein levels at passage 9 [schizophrenia 3174,70 (233,94), healthy controls 2577,12 (225,26), p = 0.007].

3.5. Cognitive performance in patients with schizophrenia and healthy controls

Results of cognitive tests in both patients with schizophrenia and healthy controls are reported in detail in Table 4. Patients with schizophrenia showed an impairment in cognitive functioning compared with healthy controls in the majority of tests. Brief neuropsychological examination [schizophrenia 73.08 (10.42), healthy controls 90.4 (5.30), p < 0.001], digit-span task [schizophrenia 5.25 (1.71), healthy controls 6.5 (0.71), p = 0.019], verbal memory at short-term [schizophrenia 9.67 (5.14), healthy controls 14.9 (5.8), p = 0.036], recall [schizophrenia 11.58 (5), healthy controls 20.9 (4.9), p < 0.001], with interference at 10 s

Table 2
Fresh ONE cell proliferation in patients with schizophrenia and healthy controls (BrdU incorporation – rlu/s (10-3)).

Cell passage	Patients Mean (Standard deviation)	Healthy controls BrdU incorporation – rlu/s (10-3)	t-test or Mann-Whitney	p value
P3				
P3 12	8.65 (0.23)	3.5 (0.25)	t 52.66, df 23	<0.001
P3 24	12.18 (0.23)	8.51 (0.27)	t 35.82, df 23	<0.001
P3 48	15.41 (0.30)	11.96 (0.25)	t 31.40, df 23	<0.001
P3 mean	12.08 (0.21)	7.99 (0.15)	t 56.43, df 23	<0.001
P6				
P6 12	4.11 (0.10)	4.05 (0.25)	t 0.709, df 21	0.486
P6 24	9.14 (0.09)	9.1 (0.17)	t 0.685, df 21	0.501
P6 48	13.86 (0.15)	13.73 (0.23)	t 1.59, df 21	0.127
P6 mean	9.04 (0.06)	8.96 (0.09)	t 2.25, df 21	0.035
P9				
P9 12 [*]	2.83 (0.1)	3.97 (0.12)	Mann-Whitney U = 0.000	<0.001
P9 24	6.55 (0.37)	9.05 (0.14)	t -20.82, df 21	<0.001
P9 48 [*]	9.19 (0.48)	14.01 (0.13)	Mann-Whitney U = 0.000	<0.001
P9 mean [*]	6.19 (0.22)	9.01 (0.8)	Mann-Whitney U = 0.000	<0.001
P12				
P12 12	2.06 (0.05)	4.04 (0.11)	t -56.80, df 21	<0.001
P12 24	5.07 (0.05)	9.04 (0.1)	t -115.70, df 21	<0.001
P12 48	7 (0.1)	14.01 (0.08)	t -181.95, df 21	<0.001
P12 mean	4.71 (0.05)	9.03 (0.04)	t -118.22, df 21	<0.001

[*] Mann-Whitney test due to non−normal distribution; ONE, olfactory neuroepithelium.

Table 3
Cyclin-D1 and p-21 protein levels in patients with schizophrenia and healthy controls.

Cell passage	Cyclin-D1 Patients Mean (Standard deviation)	Healthy controls Mean (Standard deviation)	t-test or Mann-Whitney, df, p value	P21 Patients Mean (Standard deviation)	Healthy controls Mean (Standard deviation)	t-test or Mann-Whitney, df, p value
P3						
P3 00	415.96 (124.07)	110,6923 (13,08870)	Mann-Whitney U = 0.000, <0.001	4036,833 (108,7542)	4176,750 (160,5334)	t -1.768, df 23, 0.012
P3 12	2432.46 (373.21)	860.92 (83.53)	Mann-Whitney U = 0.000, <0.001	2820,250 (62,1400)	3541,833 (107,41)	Mann-Whitney U = 0.000, 0.004
P3 24	4451.67 (418.70)	2305.96 (111.63)	t 17.83, df 23, <0.001	892,167 (72,8880)	2207,500 (112,3650)	t -24.056, df 23, <0.001
P3 48	6267.08 (401.46)	3348.35 (64.20)	t 25.90, df 23, <0.001	277,833 (54,7418)	944,583 (41,6538)	t-23.743, df 23, <0.001
P3 mean	3391.79 (224.91)	1656.48 (43.49)	t 27.318, df 23, <0.001	2006,77 (46,48)	2717,67 (21,09)	t -34.115, df 23, <0.001
P6						
P6 00	203.40 (16.36)	122.71 (9.98)	t 14.24, df 23, <0.001	4082,07 (90,29)	4072,33 (94,40)	t 0.190, df 23, 0.853
P6 12	1143.15 (44.88)	1025.46 (40.73)	t 6.44, df 23, <0.001	3109,12 (235,00)	3402,17 (105,16)	Mann-Whitney U = 3.000, 0.010
P6 24	2272.80 (54.38)	2622.83 (60.44)	t 4.04, df 23, <0.001	1644,75 (573,6366)	2216,00 (101,1677)	Mann-Whitney U = 0.000, 0.003
P6 48	3937.35 (49.68)	3593.37 (63.62)	Mann-Whitney U = 0.000, <0.001	1038,00 (67,4203)	1003,67 (77,5807)	Mann-Whitney U = 18.000, <0.001
P6 mean	2001.67 (16.55)	1841.09 (22.35)	t 18.80, df 23, <0.001	2476,31 (205,79)	2673,54 (46,82)	Mann-Whitney U = 21.000, 0.48
P9						
P9 00	116.80 (11.23)	126.36 (12.99)	t -1.61, df 23, 0.124	4130,14 (133,9841)	3735,92 (860,18)	Mann-Whitney U = 16.500, 0.52
P9 12*	1040.80 (43.13)	1109.27 (47.87)	t -3.43, df 23, 0.003	3629,00 (156,33)	3364,00 (85,55)	t 3.861, df 23, 0.003
P9 24	2068.5 (69.90)	2681.71 (70.79)	t -19.93, df 23, <0.001	2757,14 ((230,73)	2172,42 (70,31)	Mann-Whitney U = 0.500, 0.003
P9 48*	3108.20 (99.17)	3630.27 (98.78)	t -12.07, df 23, <0.001	2182,50 (536,62)	1036,17 (49,65)	Mann-Whitney U = 4.500, 0.018
P9 mean*	1583.57 (32.52)	1886.54 (30.54)	t -22.01, df 23, <0.001	3174,70 (233,94)	2577,12 (225,26)	Mann-Whitney U = 3.5000,007
P12						
P12 00	99.08 (4.48)	150.18 (16.51)	t-10.33, df 23, <0.001			
P12 12	892.67 (28.68)	1170.18 (50.52)	Mann-Whitney U = 0.000, <0.001			
P12 24	1495.25 (41.90)	2716.55 (74.21)	t -49.16, df 23, <0.001			
P12 48	2031.5 (77.02)	3698.73 (65.88)	t -55.53, df 23, <0.001			
P12 mean	1129.62 (23.77)	1933.91 (28.51)	Mann-Whitney U = 0.000, <0.001			
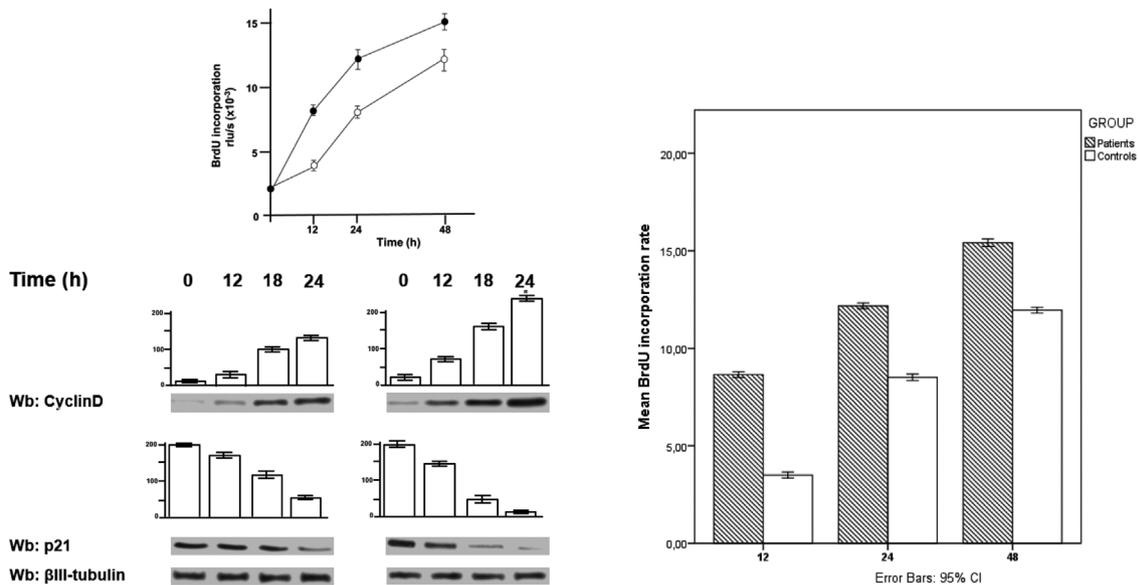
Fig. 2. Comparison of proliferation rate of fresh ONE cells of healthy controls and patients with schizophrenia at passage 3.
Legend. (Left) Proliferation of fresh ONE cells at passage 3 of one healthy control (HC5-white circles) and one patient (SZ11-black circles) was analyzed by BrdU incorporation at discrete time points for 48 h. Total lysates of fresh ONE cells at passage 3 of HC5 (left hand panels) and SZ11 (right hand panels) individuals was analyzed by Western blot analysis with antibodies against cyclin D and p21 as markers of the cell cycle at discrete time points for 24 h as well as βIII-tubulin as a loading control. (Right) Proliferation of ONE cells at passage 3 in the whole sample of patients and controls.

Table 4
Cognitive functioning in patients with schizophrenia and healthy controls.

Cognitive test	Patients Mean (Standard deviation)	Healthy controls Mean (Standard deviation)	t-test or Mann-Whitney	p value
Overall Brief Neuropsychological Examination	73.08 (10.42)	90.4 (5.30)	t -4.75, df 23	<0.001
Digit-span task[*]	5.25 (1.71)	6.5 (0.71)	U 26	0.019
Verbal memory – Short term	9.67 (5.14)	14.9 (5.8)	t -2.24, df 23	0.036
Verbal memory – Recall	11.58 (5)	20.9 (4.9)	t -4.4, df 23	<0.001
Verbal memory – with interference 10 sec[*]	7 (2.17)	8.7 (0.67)	U 29	0.025
Verbal memory – with interference 30 sec[*]	6.25 (2.8)	7.9 (1,37)	U 38	0.134
Trail Making Test – A[*]	45.17 (23.72)	23.5 (6.13)	U 10.5	0.001
Trail Making Test – B[*]	129.75 (51.26)	74.08 (30.18)	U 22	0.002
Token Motor Task[*]	4.92 (0.29)	5 (0)	U 55	0.361
Verbal Fluency	10.17 (3.1)	16.7 (2.54)	t -5.33, df 23	<0.001
Abstraction Test[*]	5.33 (1.56)	6 (0)	U 50	0.186
Cognitive Estimation Test[*]	4.83 (0.39)	4.9 (0.32)	U 56	0.658
Overlapping Figures Test	27.5 (8.18)	44.6 (3.56)	t -6.12, df 23	<0.001
Copy Drawing Test[*]	1.83 (0.39)	1.8 (0.63)	U 57	0.74
Spontaneous Drawing Test[*]	1.83 (0.39)	2 (0)	U 50	0.186
Clock Test[*]	8.75 (1.71)	9.2 (1.69)	U 50	0.423
Praxis Test[*]	5.92 (0.29)	6 (0)	U 55	0.361

[*] Mann-Whitney test due to non−normal distribution.

[schizophrenia 7 (2.17), healthy controls 8.7 (0.67), p = 0.025], trail making test A [schizophrenia 45.17 (23.72), healthy controls 23.5 (6.13), p = 0.001] and B [schizophrenia 129.75 (51.26), healthy controls 74.08 (30.18), p = 0.002], verbal fluency [schizophrenia 10.17 (3.1), healthy controls 16.7 (2.54), p < 0.001], overlapping figures test [schizophrenia 27.5 (8.18), healthy controls 44.6 (3.56), p < 0.001].

Cognitive performance did not differ between the two groups in verbal memory with interference at 30 s (p = 0.134), token motor task (p = 0.361), abstraction test (p = 0.186), cognitive estimation test (p = 0.658), copy drawing test (p = 0.74), spontaneous drawing test (p = 0.186), clock test (p = 0.423), and praxis test (0.361).

3.6. Safety and acceptability of nasal brushing

No patient asked to interrupt nasal brushing, no patient complained for relevant pain during the procedure, and no adverse event occurred during or after nasal brushing.

4. Discussion

Our results confirm that ONE fresh cells proliferation is altered in patients with schizophrenia compared with controls. [21,22] Also, we show for the first time thus advancing the knowledge in the field that such alteration is associated with cognitive impairment in patients with schizophrenia. Importantly, we also provide evidence of the peculiar proliferation alterations pattern throughout up to 12 cell passages, with patients with schizophrenia showing an initial increased proliferation, and a subsequent decreased proliferation. Moreover, we show that nasal brushing, and ONE fresh cells single-layer cultures are safe, economic, relatively fast and reliable techniques through which we replicate findings from more invasive (nasal biopsy) and complex (neurosphere) approaches [21,22].

We focused on fresh cell proliferation, but it is only one of several aspects that have been studied in ONE, with consistent findings showing significant differences in cellular structure and function between patients with schizophrenia and healthy controls, despite sample sizes as small as lower than ten individuals per group. For example, a group from Mexico has shown that ONE cells have an altered microtubule structure, [26,32] which not only differentiates from healthy controls, but also from patients with bipolar disorder. The same group also showed that primary cilia formation is altered in both patients

with schizophrenia and bipolar disorder, and that treatment with lithium in patients with bipolar disorder was associated with cilia elongation, suggesting that potential insight from ONE investigation may go beyond diagnostic improvement or prediction towards pharmacological investigation [33]. Moreover, the same group also showed that disrupted Disrupted-In-Schizophrenia-1 DISC1 pathway, a putative genetic risk factor for schizophrenia [34,35], and which underlies microtubule architecture [36], is altered in ONE cells both in schizophrenia and bipolar disorder.

Using ONE fresh cells as models of CNS diseases is advantageous compared with other sources of pluripotent stem-cells. For instance, cells for post-mortem brains are obviously not clinically useful. Also, peripheral blood cells precursors require to be differentiated into neural precursor before proliferating, with higher costs and a slower process compared with ONE cells.

Together with structural alteration in microtubules from other groups, our findings on altered cell proliferation of ONE cells in schizophrenia support the neurodevelopmental hypothesis of schizophrenia, providing empirical evidence of alteration in neural precursors in different stages of neurodevelopment, namely proliferation, and architectural organization of neurons into neural networks. Whether this alteration directly depends on an impaired upstream regulation [22] or represents a compensatory mechanism of a damage elsewhere located [37], or even a complex combination of the two, remains to be investigated.

Given the non-invasive nature of nasal brushing, the low cost, the technical accessibility and the relatively short time required to have results available ONE nasal brushing may have several clinical implications in the future. Such clinical implications are mainly related to the assumption that neurodevelopmental alteration precede clinical phenomena. First, ONE alterations may be tested as a biomarker of early stages of schizophrenia, or of At Risk Mental State (ARMS). [38] Given that olfactory identification ability has been confirmed among risk factors of psychosis [5], and that ONE underlies olfactory identification ability, it could be expected that ONE alterations are there before first-episode psychosis, for example in patients with ARMS. However, whether ONE alterations can be implemented as biomarkers of ARMS as defined by CAARMS [39], and whether they could improve ARMS's long-term prediction of transition to psychosis [38] remains to be tested in ad hoc designed prospective cohort studies. Second, ONE alterations beyond cell proliferation, namely microtubule organization may also be tested as potential predictor of development of bipolar disorder or schizophrenia specifically, given the different

Table 5
Spearman's correlation between fresh ONE cells proliferation and cognitive function in patients with schizophrenia.

	Overall	Digit Span	Verbal memory - Immediate	Verbal memory Recall	Verbal memory Interference 10 sec	Verbal memory Interference 30 sec	Trail Making Test A	Trail Making Test B	Token	Verbal Fluency	Abstraction	Cognitive estimation	Overlapping figures	Drawing copy	Spontaneous drawing	Clock test	Praxis
Cell proliferation																	
P3 12	-,563	-,279	**-,692***	**-,684***	-,537	**-,576***	-,134	,165	-,263	-,529	-,065	,163	,127	-,228	,163	-,255	,044
P3 24	**-,657***	-,393	**-,706***	**-,673***	**-,805****	**-,726****	,002	,394	-,132	-,406	,098	0,000	,069	,260	-,098	-,118	0,000
P3 48	-,023	,392	-,258	-,392	-,258	-,241	,220	-,165	-,394	-,219	0,000	-,098	,044	-,195	,228	-,291	-,307
P3 mean	-,538	-,110	**-,676***	**-,694***	**-,697***	**-,693***	,186	,210	-,393	-,464	0,000	-,065	,077	-,130	,065	-,278	,044
P6 12	,085	-,118	,055	,331	-,139	-,050	-,339	-,229	,439	,272	,358	,130	**,610***	,130	-,293	,307	,132
P6 24	,192	,114	,104	,088	,140	,166	-,406	-,002	-,265	-,174	-,196	,098	-,255	-,033	**,655***	-,382	-,486
P6 48	,296	,411	,170	,445	,214	,245	,215	-,418	,175	,503	-,228	-,098	,063	-,033	-,098	,574	,307
P6 mean	,315	,338	,084	,471	,139	,155	-,293	-,383	,133	,347	-,198	,033	,221	,033	,165	,349	-,089
P9 12	,128	-,251	-,028	,097	-,263	-,093	,198	,039	-,352	,553	-,163	**-,621***	,209	-,359	-,131	,433	,220
P9 24	,057	-,244	,227	-,124	-,158	-,020	,317	,329	,221	,213	,524	,196	-,096	,327	-,295	,146	,397
P9 48	,044	,063	-,014	,041	-,027	-,205	-,163	,219	-,353	-,286	-,033	,033	-,048	-,164	,393	-,485	-,088
P9 mean	,158	-,270	,126	-,004	-,230	-,173	,175	,389	-,131	,323	,259	-,065	-,011	,065	0,000	,157	,393
P12 12	,259	-,138	,050	-,139	-,230	,020	-,268	,050	-,133	,243	,198	,198	,020	,033	,429	,074	-,133
P12 24	-,413	**-,612***	-,359	-,355	-,065	-,226	,108	,561	-,273	-,104	-,506	-,169	-,418	-,169	,067	,173	,455
P12 48	,082	-,078	,202	,002	,154	,289	-,035	,053	-,265	-,091	-,164	,033	-,371	-,262	,327	-,257	-,088
P12 mean	,064	-,188	,117	-,064	,140	,257	-,060	,145	-,309	-,004	-,295	,065	-,438	-,295	,393	-,091	,044

* 0.01 < p < 0.05; ** p < 0.001; ONE, olfactory neuroepithelium.
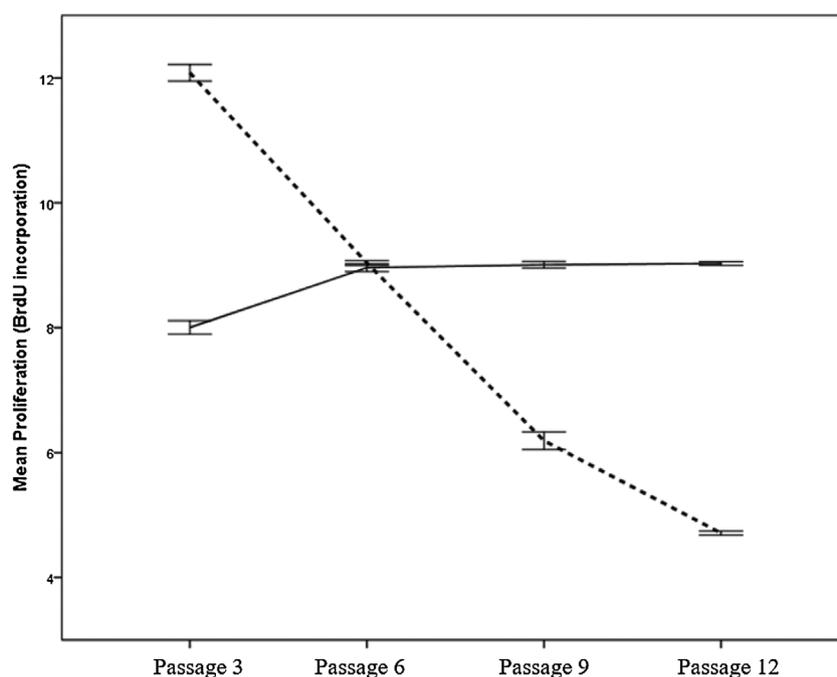
Fig. 3. Inversion of proliferation alterations of fresh ONE cells of patients with schizophrenia compared with healthy controls from passage 3 to passage 12.
Legend. Proliferation of fresh ONE cells analyzed by BrdU incorporation at passage 3, 6, 9, 12 of healthy controls (full line), and patients with schizophrenia (dashed line). Proliferation in patients is increased at passage 3, then dropping to lower levels at passage 9 and 12 compared with healthy controls.

alterations ONE has shown in the two groups in former studies [26,32]. Third, ONE could be used as a severity proxy in patients with schizophrenia before dramatic drop of functioning occurs, give that it correlates with cognitive function, and that cognitive function itself, together with negative symptoms, is strongly connected with disability and lack of social skills in schizophrenia [2,40]. Interestingly, the cognitive domain that correlated the most with ONE alterations was verbal memory, which has been shown to be altered in schizophrenia across a large body of evidence [41].

Craddock and Sadock multiple-thresholds hypothesis suggests that other severe psychiatric illnesses could fit the neurodevelopmental model; thus, a putative role of ONE alteration could be present elsewhere and be lacking of specificity for schizophrenia; the few studies conducted so far in patients with bipolar disorder [22,26,32,33] however suggest that despite some common alterations, ONE cells alterations differ between schizophrenia and bipolar disorder.

The present work as several points of strength. First, it is the first study in Europe to confirm ONE alterations in schizophrenia. Second, it is the first to correlate ONE alteration with cognitive functioning. Third, it is the second study to use nasal brushing, but the first measuring cell proliferation in ONE cells sampled with such non-invasive technique. Fourth, it investigates cell passage alterations with three different measures, namely BrdU incorporation, Cyclin-D1, p21, showing consistent results. Fifth, it is the first study to investigate ONE alterations in a sample of patients with schizophrenia diagnosed according to DSM-5 criteria. Sixth, it showed an overall excellent acceptability of the procedure, since no patient complained and no adverse event occurred. Seventh, it is the first study to show the initial increased proliferation and subsequent decreased proliferation that distinguishes patients with schizophrenia from healthy controls.

The present work as also several limitations. First, the sample size is small. Second, we did not include patients with bipolar disorder or major depressive disorder, thus schizophrenia-specificity of the results should be confirmed on further studies. Third, healthy controls had higher education compared with patients with schizophrenia. Fourth, we only provide a partial view of ONE alterations in schizophrenia, namely cell proliferation. Fifth, whether the drop in proliferation in cells from patients with schizophrenia after passage 9 is due to cell senescence or cell quiescence remains to be clarified, given the lack of any measure of putative mechanistic processes driving such a drop in proliferation in the present study. Sixth, the present findings must be considered as preliminary, and need external replication in a in independent sample.

In conclusion, olfactory neuroepithelium can be considered a biomarker distinguishing schizophrenia from healthy controls, which could be safely implemented in clinical context. Fresh cell proliferation at passage 3 can also be considered as a promising proxy of cognitive functioning in patients with schizophrenia. Further studies should test whether such findings are confirmed in earlier stages of the disease, or in patients at risk for psychosis.

Acknowledgements

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