# Human olfactory neuronal cells through nasal biopsy: molecular characterization and utility in brain science

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**ABSTRACT**

Biopsy is crucial in clinical medicine to obtain tissues and cells that may directly reflect the pathological changes of each disease. However, the brain is an exception due to ethical and practical challenges. Nasal biopsy, which captures olfactory neurons and their progenitors, has been considered as an alternative method of obtaining neuronal cells from living patients. Multiple groups, including us, have enriched olfactory neuronal cells (ONCs) from nasal biopsied tissue. ONCs can be obtained from repeated biopsies in a longitudinal study, providing mechanistic insight associated with dynamic changes along the disease trajectory and treatment response. Nevertheless, molecular characterization of nasal biopsied cells/tissue has been insufficient. Taking advantage of recent advances in next-generation sequencing technologies at the single-cell resolution and related rich public databases, we aimed to define the neuronal characteristics of ONCs, their homogeneity, and their utility. We conducted single-cell and bulk RNA sequencing, analyzed and compared the data with multiple public datasets. We observed that the molecular signatures of ONCs are similar to those of neurons, distinct from major glial cells. The signatures of ONCs resemble those of developing neurons and share features of excitatory neurons in the prefrontal and cingulate cortex. The high homogeneity of ONCs is advantageous in pharmacological, functional, and protein studies. Together, the present data solidify the utility of ONCs in studying molecular mechanisms and exploring objective biomarkers for brain disorders. The ONCs may also be useful in studying the potential link between the olfactory epithelium impairment and the resultant mental dysfunction elicited by SARS-CoV-2.

## SIGNIFICANCE STATEMENT

To study dynamic changes and underlying mechanisms along disease trajectory and treatment response in neuropsychiatric disorders, olfactory neuronal cells (ONCs) enriched from nasal biopsied tissue may provide a crucial tool. Because ONCs can be obtained from repeated biopsies in a longitudinal study, this tool has been believed to be useful and complementary to postmortem brains and induced pluripotent stem cell-derived neurons. Nevertheless, molecular characterization of nasal biopsied cells/tissue has been insufficient, which hampers a wider use of this resource. Taking advantage of recent advances in next-generation sequencing technologies at the single-cell resolution and related rich public databases, the present study defines the neuronal characteristics of ONCs, their homogeneity, and their unique utility for the first time.

## INTRODUCTION

Biopsy is an essential procedure of clinical medicine where we obtain tissues and cells that are supposed to directly reflect pathological changes of each disease. This procedure allows us to analyze pathological deficits at the molecular and cellular levels, which contributes to precise diagnosis and proper choice of medications (Joyce et al., 2012; Elston et al., 2016; Schiffer, 2018). These biospecimens are also useful in drug discovery (Basik et al., 2013; Jafari et al., 2014; Morrison, 2017; Siravegna et al., 2017; Fernández-Lázaro et al., 2020). By using such biospecimens in combination with frontline multi-omics, such as genomic, epigenomic, and proteomic approaches, many successful basic and translational research projects have expanded scientific knowledge of disease pathophysiology. The successful precedents include the Encyclopedia of DNA Elements (ENCODE) (Consortium, 2004) and the Cancer Genome Atlas (TCGA) (Tomczak et al., 2015).

Nevertheless, because of the challenges of brain biopsy at both practical and ethical levels, this approach to brain disorders is not straightforward. To overcome this limitation, a translational application of induced pluripotent stem cells (iPSCs) has been considered. iPSC-derived neurons have been successfully used in many basic studies because these neurons are expected to maintain cell-type-specific molecular signatures. A possible drawback of the iPSC-based methodology is that reprogramming in iPSC protocols could affect molecular signatures associated with disease states, which may limit the utility of iPSCs in longitudinal studies (De Los Angeles et al., 2021). In addition, the scalability of iPSCs faces some technical and budget challenges (Huang et al., 2019; Ortuño-Costela et al., 2019). Furthermore, many iPSC protocols lead to heterogeneous sets of neurons and non-neuronal cells in culture, which hampers

biochemical and pharmacological approaches that need homogeneous cell populations, although single-cell assays may complement this limitation to some extent.

Since the 1980s, the olfactory neural epithelium has also been considered as a potentially useful biospecimen for brain studies (Lovell et al., 1982; Graziadei and Monti Graziadei, 1985; Talamo et al., 1989). This epithelium, directly accessible through the nasal cavity, contains olfactory neurons and their progenitors that regenerate mature neurons throughout life (Graziadei and Graziadei, 1979). Owing to the difficulty of capturing only neural epithelium and the potential contamination of mucosal tissues in nasal biopsy, multiple groups have developed protocols to enrich olfactory neuronal cells (ONCs) from the biopsied tissue, expecting that these cells have neuronal characteristics (Mackay-Sim, 2012; Borgmann-Winter et al., 2015; Kumar et al., 2018; Rhie et al., 2018; Evgrafov et al., 2020). Our group has also published many reports using ONCs (Sawa and Cascella, 2009; Horiuchi et al., 2013; Kano et al., 2013; Mor et al., 2013; Lavoie et al., 2017; Sumitomo et al., 2018; Takayanagi et al., 2021; Namkung et al., 2023; Jaaro-Peled et al., 2022). Nevertheless, insufficient characterization of biopsied cells/tissue has limited the wider use of nasal tissues as surrogate resources for brain science.

Taking advantage of recent advances in next-generation sequencing technologies at the single-cell resolution and related rich public databases, we aimed to overcome this challenge. The goals of the present study are to define the neuronal characteristics of ONCs, their homogeneity, and their unique utility. To address these questions, we conducted single-cell and bulk RNA sequencing (RNA-seq), then analyzed and compared the data with multiple public datasets. We also utilized two cohorts of patients with psychotic disorders to explore the utility of ONCs.

## MATERIALS AND METHODS

### Nasal biopsy

Nasal biopsy was performed under endoscopic control with local anesthesia to the nasal cavity provided by lidocaine liquid 4% and oxymetazoline HCl 0.05% spray, followed by injection of 1% lidocaine and 1/100,000 epinephrine. The olfactory epithelium (OE) is more likely to be found further back in the nasal cavity on the septum as well as on the turbinates (Féron et al., 1998). More specifically, the probability of obtaining OE was reportedly 40 %, 76 %, 30-50 %, 73 %, and 58 % at the dorsomedial and the dorsoposterior areas of the septum, the dorsomedial and dorsoposterior areas of the superior turbinate, and the middle turbinate, respectively (Féron et al., 1998). Based on this information, three tissue pieces were taken from the OE regions of the septum from each nostril (6 pieces total) by using ethmoid forceps to avoid trauma to the cribriform plate. When accessing the septum was difficult, the superior turbinate was targeted. After the biopsy, participants were observed in the clinic for 15 to 30 min with no packing or treatment. One participant reported bleeding and was treated with packing among more than 400 biopsies so far.

### ONCs

**Fig. 1** summarizes the procedure for enriching ONCs from nasal biopsy. First, biopsied tissues were incubated with 2.4 U/mL Dispase II for 45 min at 37ºC, and mechanically minced into small pieces. Then, the tissue pieces were further treated with 0.25 mg/mL collagenase A for 10 min at 37ºC. Dissociated cells were gently suspended, and centrifuged to obtain pellets. Cell pellets were resuspended in DMEM/F12 supplemented with 10% FBS and antibiotics (D-MEM/F12 medium), and tissue debris was removed by transferring only the cell suspension into

a new tube. Cells from the suspension were then plated on 6-well plates in fresh D-MEM/F12 medium (Day 0 plate). Cells floating were collected on day 2 (Day 2 plate) and further incubated on 6-well plates. On day 7, the cells floating in the Day 2 plate were collected and further incubated on 6-well plates (Day 7 plate). Through these processes, connective tissue-origin cells and non-neuronal cells that are attached to Day 0 and Day 2 plates are removed. Accordingly, in the present study, we examined cells in Day 7 plates called ONCs under the hypothesis that these are neuronal and homogeneous. ONCs were aliquoted and stored in liquid nitrogen tanks for further experiments, at which point ONCs were recovered and cultured in the D-MEM/F12 medium with a media change every 2-3 days.

**Human subjects who contributed to the nasal biopsy**

The present study used clinical data and biospecimens collected from study participants recruited by clinical cohorts established at the Johns Hopkins Schizophrenia Center (Kano et al., 2013; Mor et al., 2013; Kamath et al., 2018, 2019; Posporelis et al., 2018; Faria et al., 2019, 2021; Nucifora et al., 2019; Wang et al., 2019, 2023; Avigdor et al., 2021; Coughlin et al., 2021; Etyemez et al., 2021, 2022; Narita et al., 2021; Yang et al., 2021, 2022). This study was conducted with the approval of the Johns Hopkins Medicine Institutional Review Boards. All study participants provided written informed consent. All study participants have no history of traumatic brain injury, cancer, abnormal bleeding, or other major physical conditions, no substance or alcohol abuse in the past three years, and no illicit drug use in the past two months. Healthy controls must have no personal or immediate family history of psychosis and do not take psychotropic medication at enrollment. Patients received the Structured Clinical Interview for DSM-IV (SCID-IV) by study team psychiatrists. Patients with chronic schizophrenia or first

episode psychosis (disease onset within 24 months of initial study enrollment) were used in this study. Collateral information for patients was also gathered from available medical records.

**External datasets as references for the homogeneity of ONCs.**

[1] HEK293T and Jurkat cells: single-cell RNA sequencing (scRNA-seq: 10x sequencing) was conducted in cells from American Type Culture Collection (ATCC) and cultured according to ATCC guidelines (Zheng et al., 2017).

[2] iPSC-derived neural mixed culture: scRNA-seq/10x sequencing was conducted in neural culture from iPSCs by a chemical induction (Earley et al., 2021).

[3] iPSC-derived dopaminergic neurons: scRNA-seq/10x sequencing was conducted in fluorescently activated cell sorting (FACS)-purified tyrosine hydroxylase (TH) neurons differentiated from iPSCs (Earley et al., 2021).

[4] iPSC-derived sensory neurons: scRNA-seq/10x sequencing was conducted in nociceptive sensory neurons induced from iPSCs (Plumbly et al., 2022).

[5] iPSC-derived motor neurons: scRNA-seq/10x sequencing was conducted in spinal motor neurons induced from iPSCs (Thiry et al., 2020).

[6] Postmortem subgenual anterior cingulate cortex (sACC) and dorsolateral prefrontal cortex (DLPFC): single-nucleus RNA sequencing (snRNA-seq: 10x sequencing) was conducted in tissues from healthy subjects without neurological disorders (Tran et al., 2021).

**External datasets as references for the neuronal characteristics of ONCs.**

[i] Postmortem sACC, DLPFC, hippocampus, nucleus accumbens, and amygdala: snRNA-seq/10x sequencing was conducted in the tissues from healthy subjects without neurological

disorders (Tran et al., 2021).

[ii] Postmortem prefrontal cortex: snRNA-seq/10x sequencing was conducted in the tissues from healthy subjects without neurological disorders (Velmeshev et al., 2019).

[iii] Postmortem middle temporal gyrus: snRNA-seq/10x sequencing was conducted in the tissues from healthy subjects without neurological disorders (Hodge et al., 2019).

[iv] Olfactory tissue: scRNA-seq/10x sequencing was conducted in fresh tissue samples from subjects undergoing endoscopic nasal surgery (Durante et al., 2020).

[v] Postmortem brains at different developmental stages: bulk RNA-seq was conducted in the postmortem brains at the following developmental stages: early fetus (0-12 post-conception weeks), middle fetus (13-18 post-conception weeks), late fetus (19-40 post-conception weeks), infancy (0-2 years old), childhood (3-10 years old), adolescence (11-19 years old), and adulthood (20-40 years old), in the BrainSpan project (Miller et al., 2014).

**External datasets used as quality controls for *Seurat* cell type prediction.**

*Positive controls: samples with known brain cell types*

[I] Brain neurons: snRNA-seq / HiSeq sequencing was conducted in the neuronal nuclei [enriched by using neuronal nuclear antigen (NeuN)] from postmortem brain tissues of a normal subject (Lake et al., 2016).

[II] iPSC-derived dopaminergic neurons: scRNA-seq/10x sequencing was conducted in FACS-purified TH neurons programmed from iPSCs (Earley et al., 2021).

[III] Brain microglia: scRNA-seq/10x sequencing was conducted in FACS-purified microglia from postmortem brain tissues (Gerrits et al., 2020).

*Negative control: sample with known cell types not from the brain*

[IV] Human peripheral blood mononuclear cells (PBMCs): scRNA-seq/10x sequencing was conducted in fresh PBMCs purchased from ALLCELLS (Villani et al., 2017).

**Bulk RNA-seq**

*Library preparation and sequencing*: total RNA was isolated from the ONCs using the RNeasy Plus Mini Kit (Qiagen). RNA quality was assessed via Agilent Fragment Analyzer analysis using a High Sensitivity RNA analysis kit (DNF-472) and quantified using a Qubit 4 RNA BR kit (Thermo Fisher). RNA libraries were prepared with 500 ng total RNA. Library generation was accomplished using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760 and E7490) following the NEBNext Poly(A) mRNA Magnetic Isolation Module protocol. Libraries were enriched using 11 cycles of PCR amplification. Library quality and quantification were assessed via Fragment Analyzer analysis using a High Sensitivity NGS Kit (DNF-474) and Qubit 4. Samples were then normalized to 4 nM and pooled in equimolar amounts. Paired-End Sequencing was performed using Illumina's NovaSeq6000 S4 200-cycle kit.

*Data preprocessing and analysis:* Sequencing reads were filtered to remove adapters, primers, and reads with low quality (base call accuracy < 90%) or shorter than 20 nt using *FastQC* (Andrews, 2010) and *cutadapt* (Martin, 2011), then mapped to the human genome version GRCh38 (Genome Reference Consortium Human Build 38) using *Hisat2* (Pertea et al., 2016). Count table was created after transcript assembly and abundance estimation using *Stringtie* (Pertea et al., 2016), then the batch effect was adjusted using *Combat* (Leek et al., 2012). Lowly expressed genes (gene counts were less than 10 in more than 30% of samples) were removed.

Principal component analysis (PCA) was conducted to check outliers (one outlier was identified and removed from downstream analyses). Lastly, the processed bulk RNA-seq data from healthy controls were compared with public datasets via PCA to investigate the neuronal characteristics of ONCs.

**scRNA-seq**

*Library preparation, sequencing, and data preprocessing*: 10xGenomic single cell 3′ library was prepared as follows: single cells were sorted into 1.5 mL tubes (Eppendorf) and counted under the microscope. Cells were loaded at 10,000-100,000 cells/chip position using the Chromium Single cell 3′ Library, Gel Bead & Multiplex Kit and Chip Kit (10× Genomics, V2 barcoding chemistry) and the subsequent steps were performed according to the manufacturer's instructions. After the library preparation, sequencing reads were obtained by an Illumina NovaSeq 6000 sequencer. Cell Ranger (Zheng et al., 2017) was then used to map reads to human genome hg19 (Haeussler et al., 2019), and create count tables. After this, cells with less than 200 expressed genes or higher than 6,000 expressed genes were and cells with mitochondria content higher than 5% were discarded. Lowly expressed genes [mean UMI (unique molecular identifiers per million reads) smaller than 0.1] were filtered out. Lastly, gene expression levels were normalized and log transformed for downstream analysis.

*Homogeneity assessment*: Kendall's tau, a nonparametric measure of ordinal association (Hodges, JL Jr. and Lehmann, EL, 1963), was calculated between cell pairs within the sample to estimate the intrasample homogeneity. Specifically, 10,000 cell pairs within one sample were randomly selected, then Kendall's tau of gene expression levels between cells in each pair was

calculated. The average Kendall's tau over 10,000 cell pairs was used to estimate the intrasample homogeneity. Samples with higher homogeneity are expected to have more similar expressional landscapes between cells, resulting in larger average values of Kendall's tau.

*Neuronal characteristics assessment*: we utilized snRNA-seq data from postmortem brains (see details in Methods "Datasets used as references for the neuronal characteristics of ONCs" section) to predict the cell type of ONCs using *Seurat* (Stuart et al., 2019). Specifically, anchors between the reference (snRNA-seq data from postmortem brains) and query (scRNA-seq data from ONCs) datasets were identified and used for batch effect correction. Then the cell type of query cells was predicted based on the anchor weight, a measure of the shared neighbor overlap and distance between the anchor and query cells. As a quality control, we tested this analytic pipeline on samples with known cell types, including human brain neurons, human brain microglia, iPSC-derived neurons, and human PBMCs (see details in Methods "Datasets used as quality controls for *Seurat* cell type prediction" section), to ensure that correct predictions can be provided by this analysis.

**Protein study in reference to clinical information**

*Western blotting:* ONCs were lysed and sonicated in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with protease inhibitors and phosphatase inhibitors. Cell supernatant obtained by centrifugation at 16,000 x g for 20 min at 4 °C was subjected to SDS-PAGE followed by western blotting with an anti-P62 antibody (MBL, PM066) and an anti-GAPDH antibody (Santa Cruz Biotech, sc-32233). Signal detection

was carried out with LAS 1000, and the signal intensity was quantified with Image J software. GAPDH levels were used as loading controls.

*Statistical analysis:* Analysis of covariance (ANCOVA) controlling for gender, race, and smoking status was conducted to compare the P62 levels between patients and controls. Partial Pearson correlation analysis controlling for gender, race, and smoking status was performed to study the correlations between chronological age (age in calendar years) and P62 protein levels in healthy controls.

## RESULTS

### ONCs are homogeneous

First, we asked whether ONCs are homogeneous. To address this question, we compared scRNA-seq data from ONCs of three healthy controls (see demographic summary in **Table 1**) with scRNA-seq data from immortalized cell lines (HEK293T and Jurkat cells) (Zheng et al., 2017), scRNA-seq data from iPSC-derived neural mixed culture (Earley et al., 2021), scRNA-seq data from iPSC-derived dopaminergic neurons (Earley et al., 2021), and snRNA-seq data from postmortem sACC and DLPFC from healthy subjects without neurological disorders (each individual subject was analyzed separately) (Tran et al., 2021). The immortalized cell lines were chosen as examples of homogeneous cells. Kendall's tau, which measures the similarity of expressional landscapes between cells within samples, was calculated to assess intrasample homogeneity.

Our data showed that ONCs had similar homogeneity to well-established homogeneous cell lines such as HEK293T and Jurkat cells (**Fig. 2**). The average Kendall's tau was 0.46, 0.44, and 0.42 for ONCs of each individual; similarly, Kendall's tau was 0.44 and 0.46 for HEK293T and Jurkat cells, respectively. On the other hand, iPSC-derived neurons were more heterogeneous, with an average Kendall's tau of 0.30, 0.36, 0.31, and 0.30 for iPSC-derived mixed neural culture, dopaminergic neurons, nociceptive sensory neurons, and spinal motor neurons, respectively. In the case of postmortem brain tissues with heterogeneous cell populations, the average Kendall's tau was in the range of 0.25 – 0.27. These results suggested that ONCs were close to homogeneous cell lines and more homogeneous than iPSC-derived neurons.

**Neuronal characteristics of ONCs**

Next, we asked whether the molecular signature of ONCs is neuronal. To address this question, we compared bulk RNA-seq and scRNA-seq data from ONCs with those from the brains, iPSC-derived dopaminergic neurons, and the OE tissues.

We conducted PCA analysis to compare bulk RNA-seq data from ONCs of 61 healthy controls (see demographic summary in **Table 2**) with bulk RNA-seq data from postmortem brains at different developmental stages including the fetus, infancy, childhood, adolescence, and adulthood (Miller et al., 2014). Our analysis showed that ONCs were most similar to the fetal brain (Extended Data **Fig. 3-1**).

We also utilized scRNA-seq data from ONCs of 3 healthy controls (**Table 1**) and snRNA-seq data from three independent postmortem brain datasets as references. *Seurat* predicted ONCs as

neurons against other cell types in the brain, including astrocytes, microglia, oligodendrocytes, oligodendrocyte progenitor cells, and endothelial cells, for all three reference datasets (**Fig. 3A, 3B**; Extended Data **Fig. 3-2**).

When comparing ONCs with neuronal subtypes from five different brain regions, including the sACC, DLPFC, hippocampus, nucleus accumbens, and amygdala (Tran et al., 2021), *Seurat* predicted the majority of ONCs as excitatory neurons in the sACC and DLPFC (**Fig. 3C**). UMAP (Uniform Manifold Approximation and Projection) (McInnes et al., 2018) also placed ONCs onto the space overlapped with excitatory neurons in the sACC and DLPFC (**Fig. 3D, 3E**).

Lastly, we compared scRNA-seq data from ONCs with those from the OE tissues (Durante et al., 2020). UMAP showed that ONCs were clustered in the space where horizontal basal cells gathered and far away from where pericytes and fibroblasts gathered (Extended Data **Fig. 3-3**). Olfactory horizontal basal cells are stem cells that can regenerate olfactory neurons and non-neuronal cells after injuries (Iwai et al., 2008). Interestingly, despite their origin from the OE, ONCs resemble neurons in the brain (**Fig. 3**) rather than olfactory sensory neurons in the OE (Extended Data **Fig. 3-3**).

Together, our study showed that ONCs are homogeneous cells whose molecular signatures are similar to neuronal cells, which may be a useful cell resource for studying molecular signatures of brain disorders. Homogeneous cells are advantageous in research where bulk cells are needed, such as in protein studies.

**Unique utility of ONCs in protein study**

Thus far, we have shown that ONCs are homogeneous neuronal cells by using next-generation sequencing technologies at the single-cell resolution and comparing with existing public databases, which further supports the validity of our past reports (Sawa and Cascella, 2009; Horiuchi et al., 2013; Kano et al., 2013; Mor et al., 2013; Lavoie et al., 2017; Sumitomo et al., 2018; Takayanagi et al., 2021; Namkung et al., 2023; Jaaro-Peled et al., 2022). One of these studies reported increased levels of P62 protein in ONCs from patients with schizophrenia compared with those from healthy controls (Sumitomo et al., 2018). P62 is a hub protein that plays a role in autophagy-related cellular processes (Tomoda et al., 2020). When the process is attenuated, the P62 protein level increases (Tomoda et al., 2020).

In the present study, by using a new cohort with an increased number of subjects (33 healthy controls and 30 psychotic patients, see **Table 3**) and ANCOVA analysis, we observed an upregulation of P62 protein in ONCs from patients with psychotic disorders (p-value = 3.7E-3) (**Fig. 4A**). This disease-associated protein change may be unique to neuronal cells, as we did not observe an upregulation of P62 protein in lymphoblasts from the same set of patients (**Fig. 4B**). In addition, this change may be unique to the protein levels, as we did not observe an upregulation of P62 at the mRNA levels in the same set of ONCs (**Fig. 4C**). Given that the autophagic process is attenuated with aging, we examined whether the levels of P62 in ONCs may be affected by aging via partial correlation analysis. We observed a significant, age-dependent increase in P62 protein in ONCs from healthy subjects (**Fig. 4D**). Interestingly, when we examined ONCs from patients with psychotic disorders, such an age-dependent phenotype was not observed (**Fig. 4D**). The levels of P62 were already high in young adulthood, and no

further upregulation was observed in older patients. The present study shows the reproducibility and utility of ONCs in biochemical studies that require cell homogeneity. Additionally, our findings suggest that ONCs have the potential to uncover disease-associated neuronal mechanisms.

## DISCUSSION

In the present study, we introduced a unique and easy-to-perform protocol that could establish neuronal cells from the OE. Systematic analysis using single-cell and bulk RNA-seq data showed that our culture protocol could produce homogeneous neuronal cells, which also provided strong support for many past studies that used ONCs (Sawa and Cascella, 2009; Horiuchi et al., 2013; Kano et al., 2013; Mor et al., 2013; Lavoie et al., 2017; Sumitomo et al., 2018; Takayanagi et al., 2021; Namkung et al., 2023). We propose that ONCs can be used as surrogates of neurons to address brain functions and disorders, particularly in pharmacological and biochemical studies in which the homogeneity of cells is crucial for assays.

ONCs have several additional advantages. As these are biopsied from living subjects, ONCs allow for studies linking neuronal molecular profiles to clinical manifestations (Jaaro-Peled et al., 2022). As the olfactory neural epithelium can regenerate quickly, repeated biopsies are possible. In more than one institution, studies with repeated biopsies have been approved and published (McLean et al., 2018). Repeated biopsies are particularly advantageous in clinical trials when assessing the treatment response of target molecules in neuronal cells. We can also examine the molecular changes in correlation with clinical amelioration by conducting biopsies before and after trials: for example, symptom changes and transcriptional regulation of CRMP1

in olfactory neuronal cells have been reported in patients with bipolar disorder in response to lithium treatment (Sattler et al., 2011; McLean et al., 2018). Furthermore, ONCs obtained from repeated biopsies in a longitudinal study could provide mechanistic insight associated with dynamic changes along the disease trajectory. In our psychosis cohort (Yang et al., 2022; Wang et al., 2023), the return rate of study participants is equivalent between those who experienced nasal biopsy and those who did not. This indicates that the process of nasal biopsies is not invasive to discourage study participants in clinical studies. In addition, in theory, ONCs are easy to be scaled up for large populations compared to iPSC-derived neurons, which also enhances the translational potentials of ONCs.

In neurodegenerative disease research, postmortem brains have been used as a gold standard to explore molecular and cellular changes (Nagatsu and Sawada, 2007; Hopperton et al., 2018; Li et al., 2019). However, when we study neurodevelopmental disorders, we need to pay attention to at least two points: first, different from neurodegenerative diseases, pathophysiological processes may be active many years prior to an individual's death, and may not remain after death. In psychiatric disorders of neurodevelopmental origins, such as schizophrenia, long-term medication may modify intrinsic molecular signatures (Rapoport et al., 2012; Birnbaum and Weinberger, 2017). To overcome these limitations associated with postmortem brain studies for neurodevelopmental disorders, ONCs through nasal biopsy in early active stages of the diseases may be particularly useful.

Accordingly, ONCs and related neuronal cells from nasal biopsy have been published more frequently in research for psychotic disorders, such as schizophrenia (Fan et al., 2012; Kano et

al., 2013; Mor et al., 2013; Rhie et al., 2018; Sumitomo et al., 2018; Evgrafov et al., 2020; Takayanagi et al., 2021; Namkung et al., 2023). Although full consensus has not been obtained yet, given the relatively small sample sizes of each past study, common pathways such as cell cycle control, calcium signaling, and immune/inflammatory pathways have been highlighted. One potential caveat is that the pathological changes of ONCs can reflect not only those that underlie neuronal cells in general in the patients (the rationale for using ONCs as a surrogate of brain neurons) but also those that may additionally be emphasized in the nasal cavity. For example, significant changes in the immune/inflammatory pathways in ONCs of psychotic patients (Yang et al., 2021) may be a converged outcome of intrinsic susceptibility to these pathways in patients with an additional local pathology elicited by air population and viral infection (risk factors for these diseases). Nevertheless, the pathological signatures defined in ONCs can be essential clues for deciphering the molecular pathophysiology of the diseases. ONCs may also be useful in studying the potential link of the OE impairment to resultant mental dysfunction elicited by SARS-CoV-2.

We also acknowledge that, compared to postmortem brain and iPSC-derived neurons, ONCs have several limitations. Although one group has published calcium signaling deficits in nasal biopsied olfactory primary neurons from patients with bipolar disorder (Hahn et al., 2005), ONCs and related cells do not show clear action potentials seen in mature neurons. ONCs are not suitable for studying characteristics of mature and differentiated neurons. Recent advances in iPSC-based neurobiology have overcome these limitations. In summary, by conducting molecular characterization of ONCs using frontline next-generation sequencing techniques, we

describe both advantages and limitations of ONCs, and propose their unique utility that can complement the use of iPSC-derived neurons and postmortem brains.

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## Legends

**Figure 1. Experimental procedure for enriching ONCs from nasal biopsy**

**Figure 2. The homogeneity characteristics of ONCs.**

Kendall's tau was calculated to assess the intrasample homogeneity. ONCs from three donors were compared with scRNA-seq data from homogeneous cell lines (HEK293T and Jurkat cells), scRNA-seq data from iPSC-derived cells including neural mixed culture (iPSC_neuron), dopaminergic neurons (iPSC_DN), nociceptive sensory neurons (iPSC_SN), and spinal motor neurons (iPSC_MN) and snRNA-seq data from postmortem brain tissues (individual subjects were analyzed separately: sACC_donor1, sACC_donor2, DLPFC_donor1, DLPFC_donor2)

**Figure 3. The neuronal characteristics of ONCs.**

The cell type of ONCs was compared with human brain cell types in the sACC (**A**) and DLPFC (**B**) using *Seurat*. Publicly available data from brain neurons, iPSC-derived dopaminergic neurons, and brain microglia were used for quality control. The heatmap shows the percentage of cells in the tested samples (y-axis) against the reference cell types (x-axis). *Seurat* also predicted the cell type of ONCs against excitatory (EN) and inhibitory (IN) neurons across five different brain regions: sACC, DLPFC, hippocampus (HP), nucleus accumbens (NAc), and amygdala (AMY) (**C**). Furthermore, we visualized the projection of ONCs on the 2D space of brain cells in the sACC (**D**) and the 2D space of neuron subtypes in five brain regions (**E**) via UMAP.

**Figure 4. P62 and psychotic disorders.**

We compared the P62 protein levels in ONCs between healthy controls (n=33) and psychotic patients (n=30) and observed a significant increase in patients (**A**). On the other hand, the P62 protein level in lymphoblasts was similar between controls (n=32, one control didn't donate lymphoblasts) and patients (n=30) (**B**). Also, we didn't observe a significant difference in the P62 mRNA levels in ONCs between controls (n=16, we didn't have mRNA data from 17 controls) and patients (n=18, we didn't have mRNA data from 12 patients) (**C**). Lastly, we examined the correlation between the P62 protein level in ONCs and age (**D**). We observed a significant positive correlation in controls (n=33), but not in patients (n=30). Symbol * denotes significant results (p-value < 0.05).

**Table 1. Demographic summary of healthy controls used for studying the homogeneous and neuronal characteristics of ONCs at the scRNA-seq data level.**

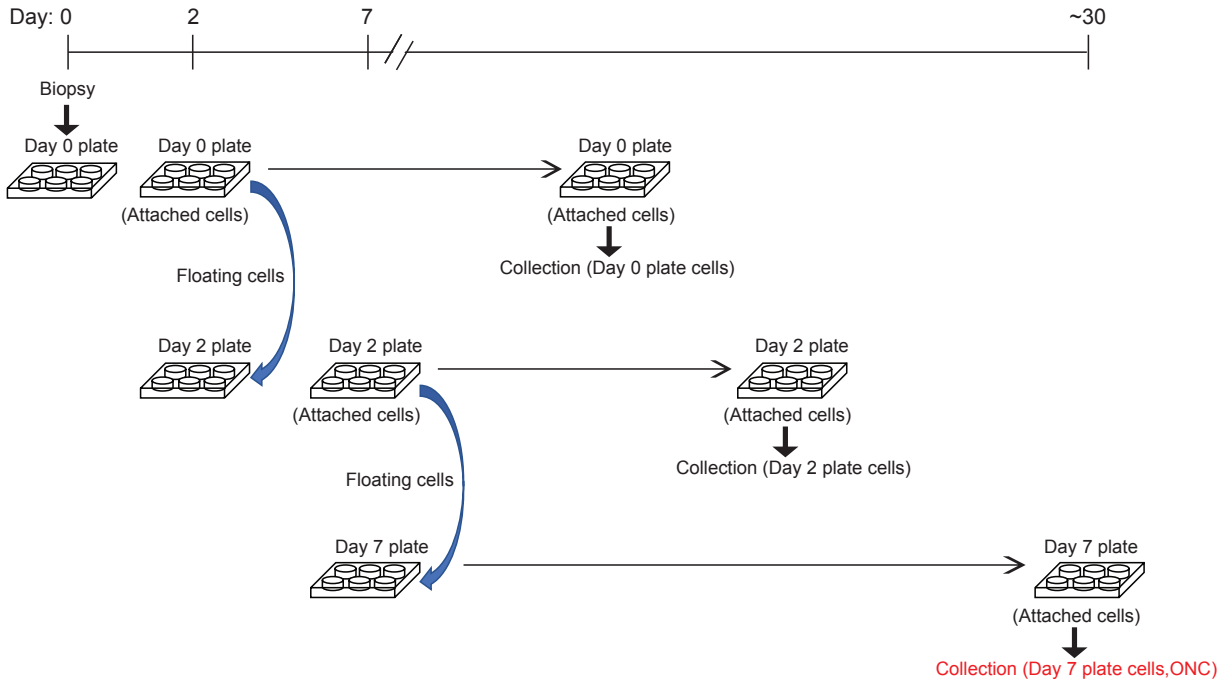
**Table 2. Demographic summary of the cohort used for studying the neuronal characteristics of ONCs at the bulk RNA-seq data level.**

Abbreviations: SD, standard deviation; Y, yes; N, no; AA, African American.

**Table 3. Demographic summary of the cohort used for studying P62 levels.**

Abbreviations: SD, standard deviation; Y, yes; N, no; AA, African American.

**Figure 1.**


Day: 0
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Collection (Day 0 plate cells)
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Day 2 plate
(Attached cells)
Day 2 plate
(Attached cells)
Collection (Day 2 plate cells)
Floating cells
Day 7 plate
Day 7 plate
(Attached cells)
Collection (Day 7 plate cells,ONC)

**Figure 2.**

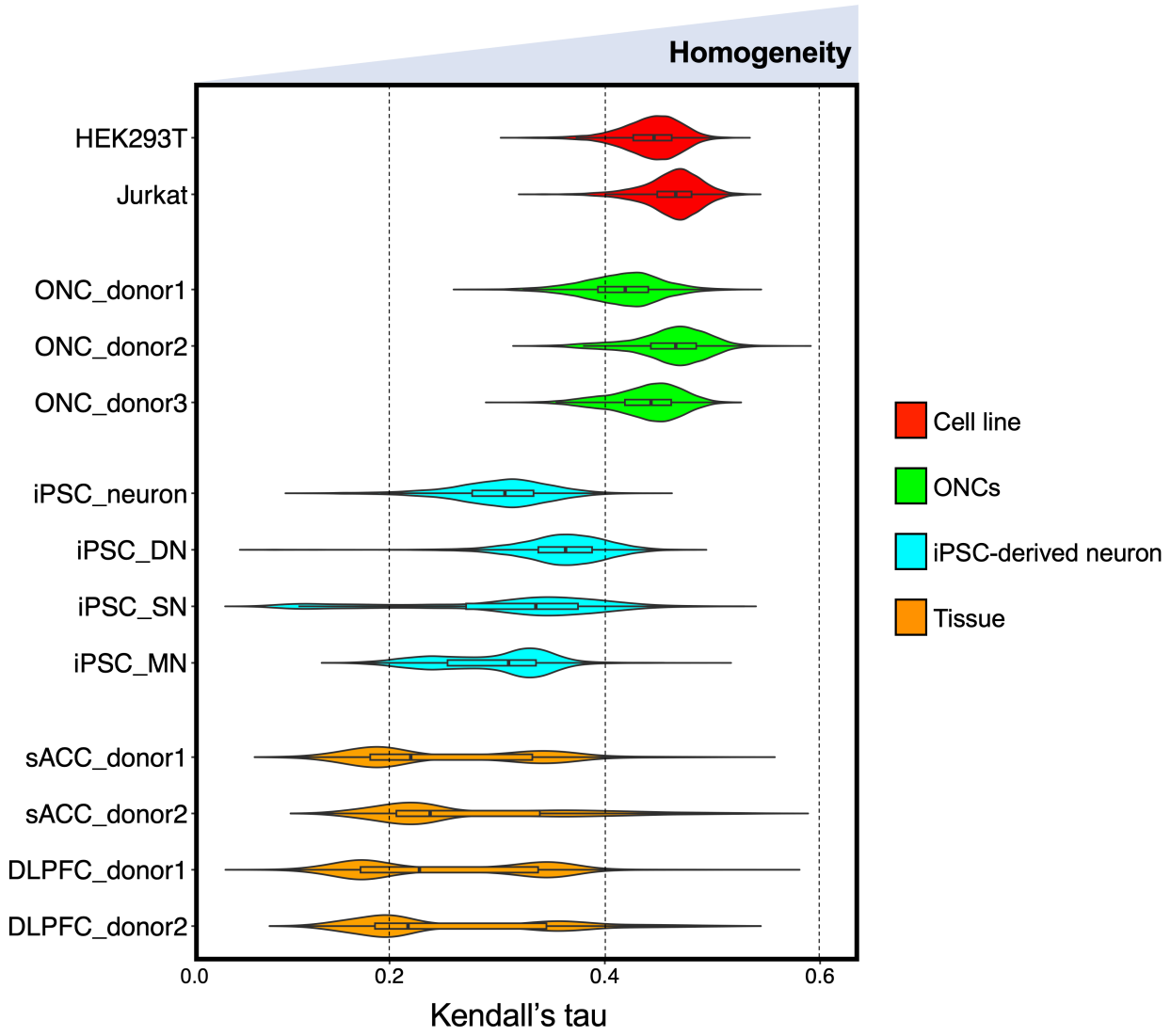
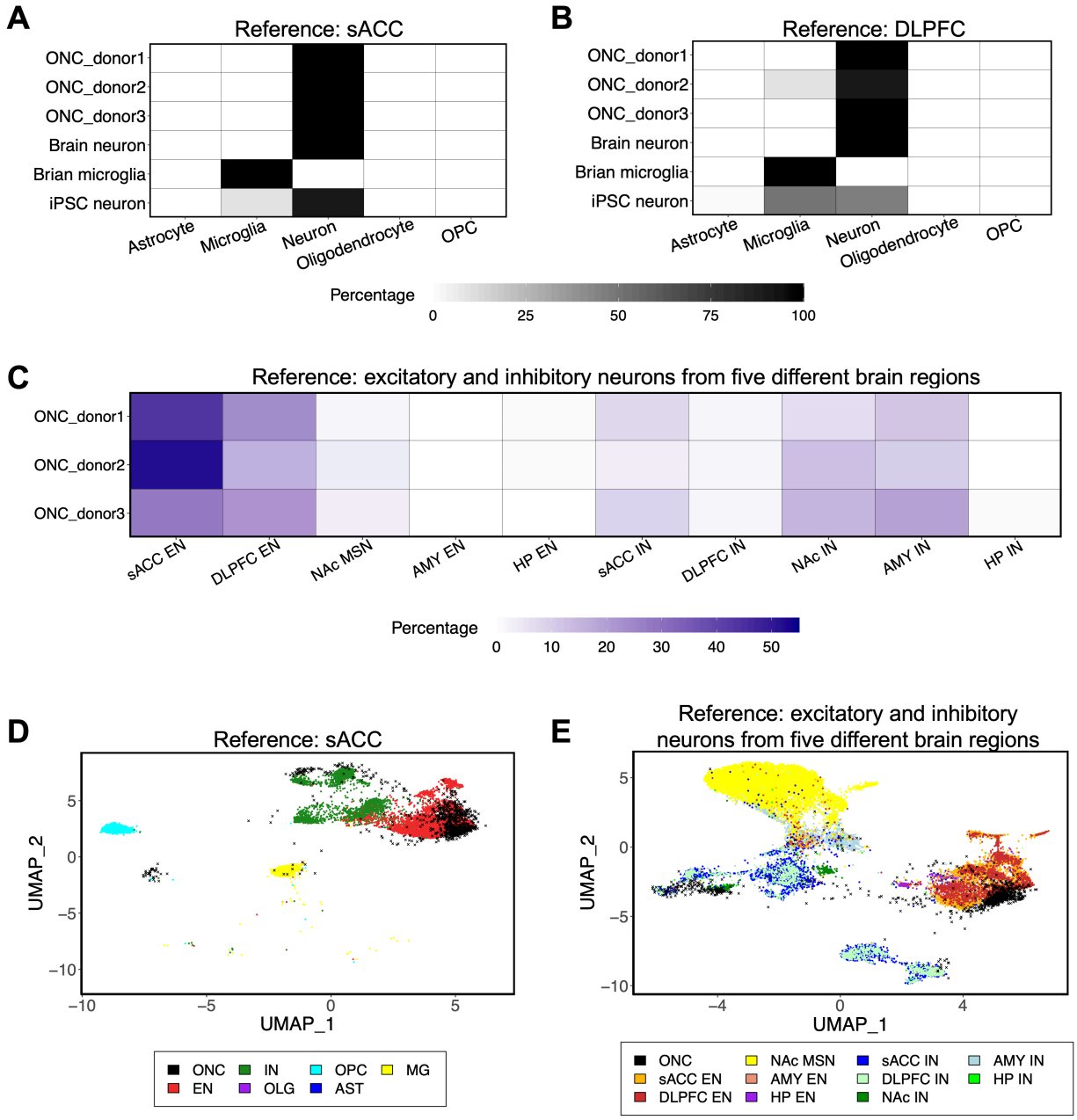

Homogeneity
HEK293T
Jurkat
ONC_donor1
ONC_donor2
ONC_donor3
iPSC_neuron
iPSC_DN
iPSC_SN
iPSC_MN
sACC_donor1
sACC_donor2
DLPFC_donor1
DLPFC_donor2
0.0
0.2
0.4
0.6
Kendall's tau
Cell line
ONCs
iPSC-derived neuron
Tissue

**Figure 3.**


A
Reference: sACC
ONC_donor1
ONC_donor2
ONC_donor3
Brain neuron
Brian microglia
iPSC neuron
Astrocyte
Microglia
Neuron
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OPC
B
Reference: DLPFC
Percentage
0
25
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Reference: excitatory and inhibitory neurons from five different brain regions
sACC EN
DLPFC EN
NAc MSN
AMY EN
HP EN
sACC IN
DLPFC IN
NAc IN
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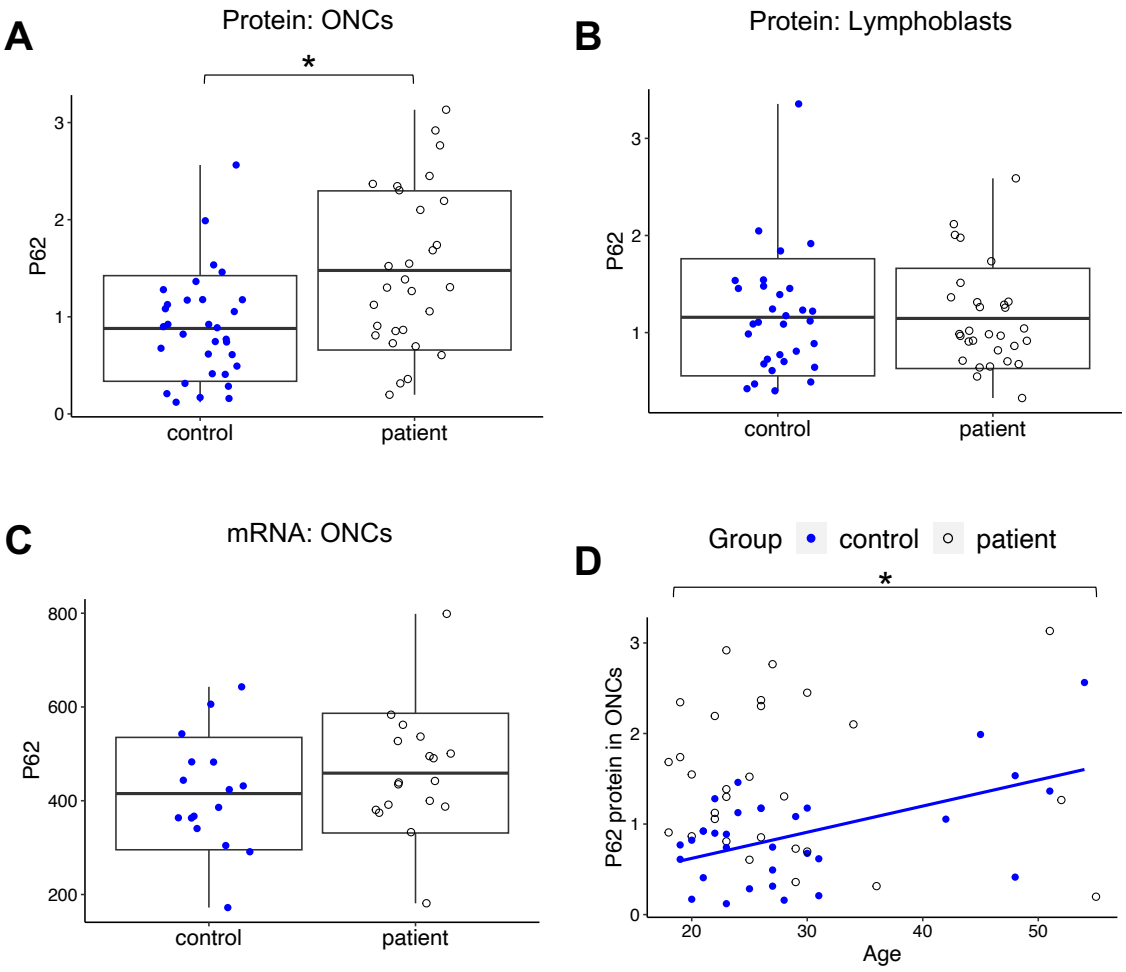
**Figure 4.**


A
Protein: ONCs
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Protein: Lymphoblasts
P62
1
2
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C
mRNA: ONCs
P62
200
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D
Group control patient
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P62 protein in ONCs
0
1
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20
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50
Age

**Table 1.**

| Age | Sex | Race | Tobacco use |
|---|---|---|---|
| 19 | Male | African American | No |
| 23 | Male | African American | No |
| 26 | Male | African American | No |

**Table 2.**

| | Controls (n=61) |
|---|---|
| Age (mean/SD) | 23.28 / 3.17 |
| Gender (Male/Female) | 31 / 30 |
| Race (AA/Caucasian/Asian/others) | 39 / 16 / 2 / 4 |
| Tobacco use (Y/N) | 2 / 59 |

**Table 3.**

| | Controls (n=33) | Patients (n=30) | p-value |
|---|---|---|---|
| Age (mean/SD) | 28.79 / 9.93 | 27.90 / 9.71 | 0.72 |
| Gender (Male/Female) | 22 / 11 | 20 / 10 | 1.00 |
| Race (AA/Caucasian/Asian/others) | 21 / 10 / 1 / 1 | 21 / 8 / 1 / 0 | 0.78 |
| Tobacco use (Y/N) | 7 / 26 | 16 / 14 | 0.02 |