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## Obtaining information from the brain in a non-invasive way: determination of iron in nasal exudate to differentiate hemorrhagic and ischemic strokes

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### Abstract

**Background:** Differentiation between hemorrhagic and ischemic stroke is currently made by brain imaging or analyzing blood and cerebrospinal fluid (CSF) samples. After describing a new drainage route from brain to nasal mucosa, nasal exudate samples can be considered a new and promising source of biomarkers. Saliva can also be evaluated.

**Methods:** We determined iron in nasal exudate and saliva samples from patients of acute stroke during the first 48 h from onset. A simple, non-invasive sampling procedure was employed to obtain information from the brain. Samples were taken with a pre-weighed swab, solved in a 2% nitric acid solution and iron was measured by inductively coupled plasma-tandem mass spectrometry (ICP-MS/MS).

**Results:** A significant difference in the dispersion of results of iron concentration for both stroke subtypes was observed in nasal exudate samples. The interquartile range was 0.608 nmol mg<sup>-1</sup> of iron for hemorrhagic strokes and only 0.044 nmol mg<sup>-1</sup> for ischemic strokes. In saliva samples, however, the values were 0.236 vs. 0.157 nmol mg<sup>-1</sup>. A cut-off limit of 0.102 nmol of iron per mg of nasal exudate provides a methodology with a 90% of sensitivity and a 90% of specificity. The value of the area

under (AUC) the receiver operating characteristic curve (ROC) for nasal exudate samples is 0.960, considered as very good in which regards to its predictive value.

**Conclusions:** Non-invasive samples of nasal secretion have allowed obtaining, for the first time, information from the brain. Determination of iron in nasal exudate by ICP-MS allowed differentiation between ischemic and hemorrhagic strokes.

**Keywords:** brain information; differential stroke diagnosis; inductively coupled plasma-tandem mass spectrometry (ICP-MS/MS); iron determination; nasal exudate samples.

## Introduction

The brain has been traditionally considered as a highly immune-protected organ. Therefore, it has been always a challenge to find biomarkers reflecting the damage of the central nervous system (CNS). Blood and cerebrospinal fluid (CSF) have been considered the main sources of information, but the need of lumbar puncture for obtaining CSF has made blood the fluid of choice in most of the studies. Recently, the description of the cerebral lymphatic drainage system, showing vessels draining to the nostrils and cervical lymph nodes [1–3], opened up new opportunities for obtaining information from the CNS. The olfactory nerve, which crosses the cribriform plate at the base of the ethmoid bone, leaves room for releasing soluble species and cells [4]. The presence of this main drainage route motivated us to examine nasal exudate samples to determine, for the first time, biomarkers of brain damage; more specifically, to differentiate hemorrhagic and ischemic stroke subtypes.

The differential diagnosis of stroke is a significant example of must-know CNS damage as: (i) stroke is always among the most prominent causes of worldwide mortality as indicated in a 2016 WHO report [5], (ii) it is essential to get the right treatment since ischemic stroke benefits from intravenous thrombolysis, which is contraindicated in hemorrhagic stroke, (iii) the quality of life of survivors depends on the time from stroke onset

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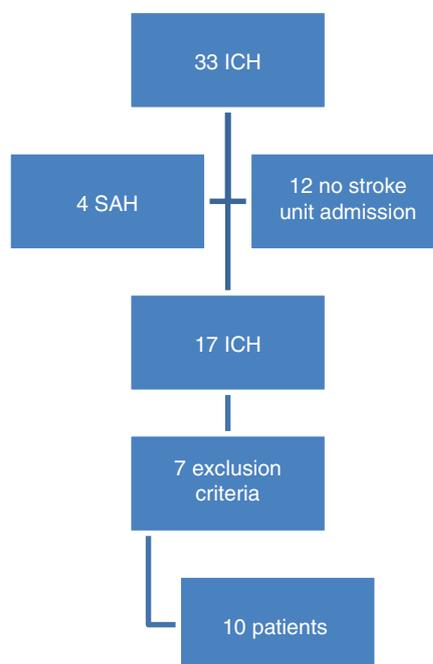
to treatment. Although the median time from symptom onset to admission in a stroke unit was 180 min for studies performed in developed areas [6], there is an urgent need for decentralized diagnosis, especially in remote locations. Therefore, identification of useful and easily accessible biomarkers is of paramount relevance. Nowadays, differentiation is mainly based on data obtained from computed tomography or magnetic resonance imaging [7], only available in main hospital settings. On the other hand, several species such as proteins delivered after brain damage, factors released after initiation of inflammation or coagulation processes or, more recently, miRNA [8–10] are analyzed in blood to help in the differential diagnostics.

Iron is an important constituent in brain and in certain regions it reaches concentrations equivalent to those in liver. It has an important role in electron transfer and is a cofactor for certain enzymes [11]. It has been described that brain injury after intracerebral hemorrhage is due in part to the release of iron from hemoglobin and studies are being performed to clarify the mechanisms of clearance [12]. In this context, we considered that iron could be a potential biomarker, able to differentiate the occurrence of hemorrhage or ischemia. Although biomarkers are currently searched for in venous blood, or very invasively in CSF, we envisioned the recently described drainage [1, 2] to the nasal mucosa as the fascinating journey biomarkers make from brain to lymph and blood. We hypothesized we could intercept them at the upper part of the nostrils to extract brain information. In this way, nasal exudate could be an excellent fluid to determine brain damage biomarkers, being the differentiation between hemorrhagic and ischemic strokes one extremely useful example.

## Materials and methods

### Human volunteers and biological sample collection

Samples of nasal exudate and saliva were taken from 10 successive patients with hemorrhagic stroke and 10 patients with ischemic stroke and the same basal characteristics, admitted in the Stroke Unit of the Central University Hospital of Asturias (HUCA) during the first 48 h from the stroke onset in order to determine iron concentration. The flow diagram for patients with hemorrhagic stroke is included in Figure 1. All the patients were evaluated neurologically (following the National Institutes of Health Stroke Scale) and computed tomography was used to determine location and extension of the vascular damage. Vitamin or mineral supplement intake, nasal hemorrhage, liver disorders or having worked in heavy industry were considered exclusion criteria. Samples were taken according to Helsinki recommendations following a consented planned protocol, using  $\delta$ SWAB swabs and sterile tubes (both from Deltalab, Barcelona,



**Figure 1:** Flow diagram corresponding to patients of stroke.

Spain, <http://www.deltalab.es/>). For sample conservation and dilution, 2% HNO<sub>3</sub> was used.

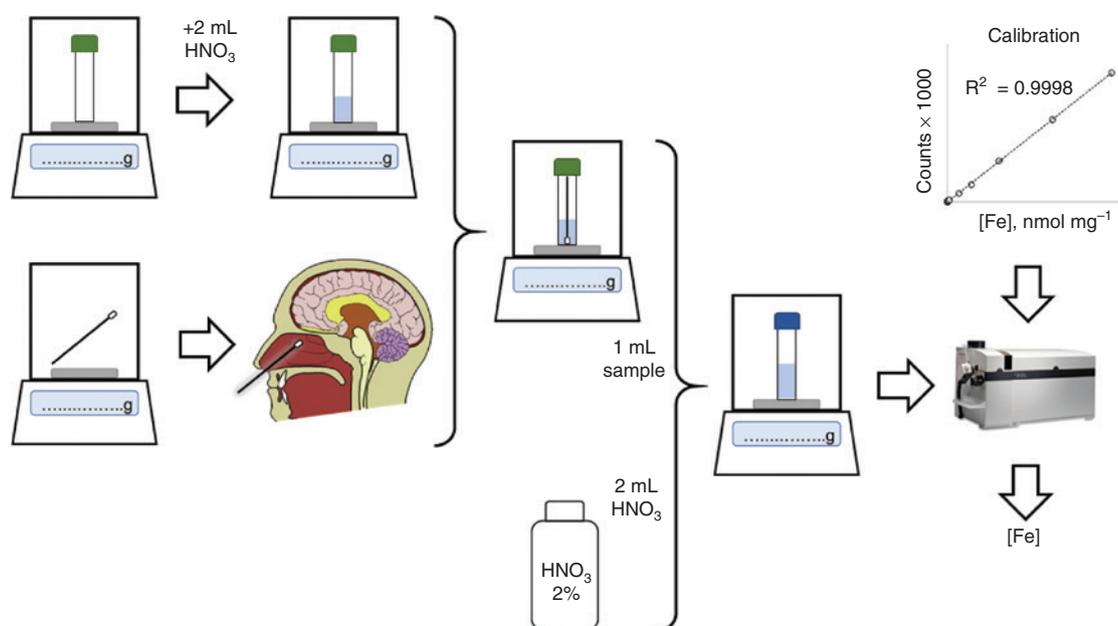
In Figure 2, a schematic of the sampling protocol is shown. Samples were taken with flocked nasopharyngeal swabs (stable in a 2% nitric acid solution). A swab and a tube containing 2 mL of a 2% nitric acid were accurately weighed (with four significant digits). Then, for taking the sample of nasal exudate, a swab was introduced successively in both nostrils, performing circular movements for 3 s in the area close to the superior turbinate. Afterwards, the swab was introduced in the tube with nitric acid and weighed again. Then, it was firmly closed and kept under refrigeration until analysis. In the case of saliva samples, swabs were passed gently by the tongue surface and the inner part of the cheeks. Later, they were treated similarly to nasal exudate samples.

### Chemicals

A standard solution of iron (1000  $\mu\text{g mL}^{-1}$ ) in 2% of nitric acid was purchased from Spex-Certiprep (Stanmore, UK). Optima grade nitric acid, special for ultratrace metal analysis (Optima Grade) was provided by Fisher Scientific ([www.fishersci.es](http://www.fishersci.es), Madrid, Spain). Water used throughout this work was obtained from a Millipore Direct-Q™ 5 purification system (Merck Millipore, Darmstadt, Germany).

### Preparation of nasal exudate and saliva samples

Both nasal exudate and saliva samples stored under refrigeration in a 2% nitric acid solution were 1:2 diluted in 2% nitric acid. This was the only sample treatment required before measurement of iron concentration by inductively coupled plasma-tandem mass spectrometry (ICP-MS/MS).



**Figure 2:** Schematics of the non-invasive sampling procedure and the rest of the analytical process.

### ICP-MS/MS method for the determination of iron in nasal exudate and saliva samples

Iron was determined using inductively coupled plasma mass spectrometry (ICP-MS) using a Triple Quad (Agilent 8800 ICP-QQQ, Tokyo, Japan, <https://www.agilent.com/>) with a concentric nebulizer with double-pass glass spray chamber Scott type. The isotope measured was  $^{56}\text{Fe}$ . The concentration was determined by external calibration. With the aim of ensuring the measurement conditions were adequate, the calibration was performed prior to the measurement of the samples. Ten different dilutions of a  $1000 \mu\text{g mL}^{-1}$  ( $179 \text{ mmol L}^{-1}$ ) iron standard solution were prepared in a wide range of concentrations using 2% nitric acid. Operation conditions for the

ICP-MS analysis were optimized using a tuning solution and the conditions described in Table 1, using helium (He) as a collision gas to suppress the signal of  $\text{ArO}^+$  interfering with the determination of Fe at  $m/z=56$ . Moreover, adding He to the collision cell of the ICP-MS was already demonstrated to be highly effective in the removal of several matrix-derived polyatomic interferences including  $^{40}\text{Ca}^{16}\text{O}$  and  $^{40}\text{Ca}^{16}\text{OH}$  that are major interferences for the determination of  $^{56}\text{Fe}$  and  $^{57}\text{Fe}$ , respectively. This, in turn, means that determination of isotope  $^{56}\text{Fe}$  in aqueous samples, that could not previously be used for iron quantification due to interferences, can be accurately measured when working in He collision mode conditions [13]. The flow of He was optimized to maximize the sensitivity.

Equipment optimization and external calibration are performed prior to every analysis to ensure that instrumental conditions do not affect the measurements. For each sample, the equipment performs 10 measurements, with 100 mass/charge measurements each replicate. The final signal, therefore, corresponds to the average of 1000 measurements.

**Table 1:** ICP-MS experimental conditions employed for iron determination in nasal exudate and saliva samples.

Parameter (Agilent 8800 ICP-QQQ)	Condition
RF power, kW	1550
RF matching	1.8
Nebulizer gas, L/min	1.1
Collision gas type	He
Collision gas, mL/min	8.0
Acquisition mode	Spectrum
Dwell time, s	1
Q2 peak pattern	3
Number of replicates	10
Sweeps/replicate	100
Nebulizer type	Concentric
Spray chamber	Double-pass glass spray chamber Scott type

He, helium; RF, radio frequency.

### Statistical analysis

Linear regression with ordinary least squares was used to calculate the fit coefficients. For each standard concentration (C), measurement of the counts for the isotope 56 of iron was considered as the analytical signal (S). A linear relationship ( $S = \text{intercept} + \text{sensitivity} \cdot C$ ) was obtained. The concentration units considered were  $\text{nmol mg}^{-1}$ , as samples were in the range of milligrams. If nmols want to be converted into ng, the elemental atomic mass of iron,  $55.85 \text{ ng nmol}^{-1}$ , should be employed. Blank samples are measured as point zero of the calibration and in between every four samples. The calibration is adjusted by subtracting the signal of the blank for all samples, so the coordinates of this point are (0,0). For sample analysis, the mean of all measured blanks is subtracted. This results in a modified linear relationship such as ( $S = \text{sensitivity} \cdot C$ ).

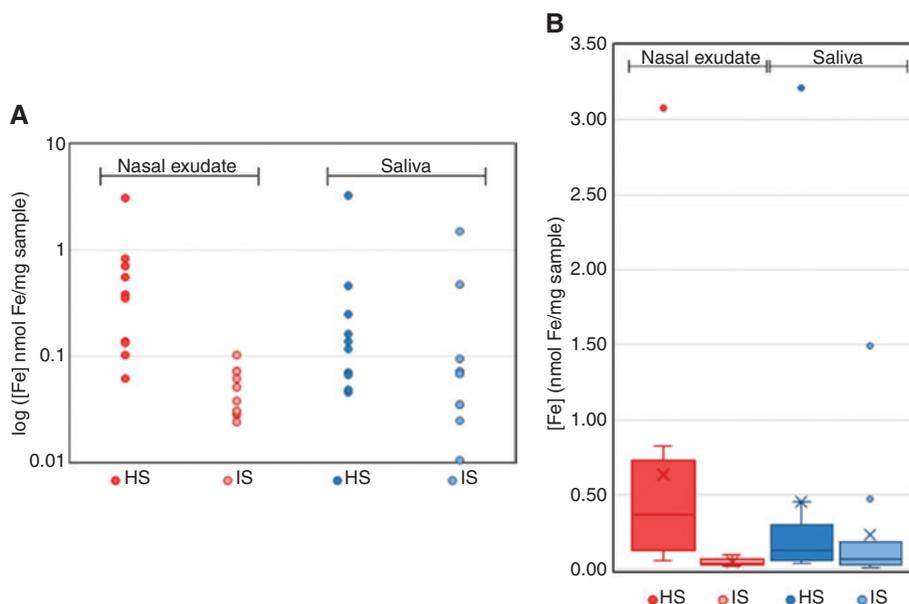
## Results

A total of 20 patients of acute stroke were enrolled in this study. The diagnosis of 12 female and 8 male patients was confirmed by computed tomography of the brain. They ranged from 61 to 83 years of age (mean value of 72 years). Based on the evaluation, 10 participants were classified as ischemic and other 10 as hemorrhagic stroke patients. All the subjects or their relatives were informed about the study and written consent was obtained before sampling. The protocol was in accordance with the Helsinki Declaration and was approved by the local Ethic Committee. Patients were recruited successively and those having some of the exclusion criteria were not considered. Nasal secretion as well as saliva samples were taken from all the patients using nasopharyngeal swabs and were blindly analyzed to determine iron by ICP-MS/MS. The weight of the nasal secretion ranged from *ca.* 2 to 60 mg, and in the case of saliva samples it ranged from *ca.* 0.4 to 80 mg. Immediate transfer to a 2% nitric acid solution (special reagent grade to avoid metal contamination) is made. A calibration curve was first performed with external iron standards (in 2% HNO<sub>3</sub>) after adjusting operational parameters (see Table 1). The coefficient of determination ( $R^2$ ) was always equal or higher than 0.998. The intercept and slope values were employed for determination of iron in samples. The limit of detection (LOD), calculated as the concentration corresponding to a signal that is 3 times the standard deviation of the blank resulted to be 9 nmol L<sup>-1</sup>. The limit

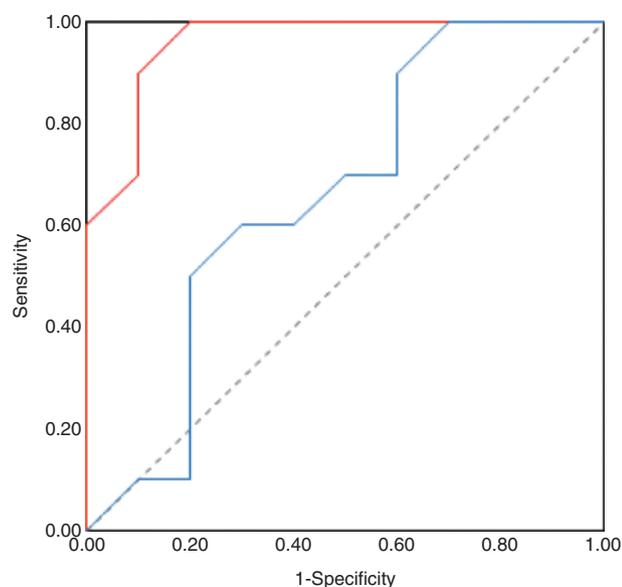
of quantification (LOQ, in this case corresponds to a signal that is 10 times the standard deviation of the blank) was 30 nmol L<sup>-1</sup>.

All iron nasal exudate and saliva measurements were above the defined LOQ of the assay. A certified reference material (iron in nasal exudate/saliva) is not available, so it could not be employed for validation. However, we consider that differential measurements (hemorrhagic vs. ischemic) would compensate possible matrix effects.

Figure 3A shows the scatter plot of the results obtained for iron concentration in a logarithmic scale. The corresponding Box-and-Whisker diagrams are presented in Figure 3B (linear scale). The average iron concentration for saliva samples in hemorrhagic strokes is 0.457 nmol mg<sup>-1</sup>, meanwhile for the ischemic subtype is 0.237 nmol mg<sup>-1</sup>. Two atypical values were detected in the ischemic group (with values of 1.492 and 0.470 nmol mg<sup>-1</sup> of sample) and one in the hemorrhagic (as high as 3.215 nmol mg<sup>-1</sup>). In both subtypes the value of the median is closer to the first quartile value but this of patients that suffered a hemorrhagic event is higher. The interquartile range, which represents the 50% of the results, was 0.236 and 0.157 nmol mg<sup>-1</sup> for hemorrhagic and ischemic strokes, respectively. In the case of the nasal secretion, the interquartile range is only 0.044 nmol mg<sup>-1</sup> for the ischemic group, and 0.608 nmol mg<sup>-1</sup> for the hemorrhagic. There is no any outlier in the ischemic group and one was found (3.077 nmol mg<sup>-1</sup>) in the hemorrhagic subtype. The average iron concentration for nasal secretion samples in



**Figure 3:** Plots indicate the dispersion of iron concentration in nasal exudate and saliva samples for acute (hemorrhagic and ischemic) stroke. (A) Dispersion curves (log scale) and (B) Box-and-Whisker plots for iron concentration, determined by ICP-MS in hemorrhagic and ischemic strokes (HS and IS, respectively) in non-invasive nasal exudate and saliva samples.



**Figure 4:** ROC curves showing the experimentally determined sensitivity-specificity relationship for the differential determination of hemorrhagic vs. ischemic strokes by ICP-MS-based detection of the concentration of iron in nasal exudate (red) and saliva samples (blue).

hemorrhagic strokes is  $0.633 \text{ nmol mg}^{-1}$ , while the average value for ischemic stroke is as low as  $0.051 \text{ nmol mg}^{-1}$ . The two lowest values for the hemorrhagic stroke are  $0.063$  and  $0.102 \text{ nmol mg}^{-1}$  and the two highest values for the ischemic events are  $0.102$  and  $0.074 \text{ nmol mg}^{-1}$ . Cross-contamination of nasal exudate or saliva samples was ruled out both during the sampling (by immediate solution of the sample in nitric acid), and during the measurements (by the cleaning of the system in between samples using freshly-diluted nitric acid). There were no statistically significant differences between genders or age groups.

Figure 4 shows the receiver operating characteristic curve (ROC) obtained for both, saliva and nasal secretion samples. Sensitivity (ratio of true positive values vs. total positive values) was plotted against (1-specificity), that is proportional to the number of false positive values. A cut-off limit of  $0.102 \text{ nmol}$  of iron per mg of sample provided a methodology with a 90% of sensitivity and a 90% of specificity. The area under the curve (AUC) had a value of 0.665 for saliva samples, but the value for nasal exudate samples was as high as 0.960.

## Discussion

Analytical chemistry is one of the branches of Chemistry that is evolving more rapidly to provide interesting

information, based on the accurate monitoring of species of interest. In this context, the decentralization of analysis is increasing exponentially to meet the requirements of rapid *in situ* analyses. Differential diagnosis of stroke is one of the areas where this trend could find an extremely useful application. However, the convenience of the use of specific biomarkers has to be first and accurately confirmed, which is usually made, as in this case, with robust centralized techniques.

According to the recent description of the lymphatic brain drainage, we hypothesized that nasal exudate could be a source of biomarkers that travelled unnoticed from brain to the cervical lymph nodes. On the other hand, although saliva is a fluid secreted by minor and major salivary glands, it has to be considered that: (i) nasal and oral cavities are related, so saliva could be a vehicle of clearance of nasal secretion; and (ii) specialized cells in glands take species from blood, mix them with saliva-specific proteins and secrete the resultant cocktail. Some substances may also reach saliva by passing from blood, and therefore compounds present in blood can be also found in saliva [14]. Thus, saliva samples were also analyzed. Both fluids, nasal exudate and saliva, allow non-invasive sampling, being far easier and cheaper to collect than blood (and CSF [15]), with no exposition to blood-borne diseases. Moreover, they are also simpler to handle because they do not clot, and less manipulation is required. We also considered that iron, present in the brain in high concentration, could carry information about stroke events. Nasal secretion and saliva samples were analyzed to determine iron concentration and to compare its value for both stroke subtypes. Here we show that iron can be reliably detected by ICP-MS/MS.

From the results, it can be concluded that: (i) the dispersion in saliva samples is very similar for both subtypes of stroke. Values of the interquartile ranges are about 1.5 times higher in the case of the hemorrhagic stroke. However, (ii) the difference in the dispersion for both subtypes of stroke is, surprisingly, very significant for nasal exudate samples. The interquartile range in this case is less than 10 times lower in ischemic strokes. (iii) Moreover, the values for iron concentration are lower for ischemic strokes in both types of samples. The average values in saliva for ischemic events present around half the value obtained for hemorrhagic strokes. In nasal exudate, the average value for ischemic stroke is notably low (less than 1/10th the average value for hemorrhagic stroke). (iv) Differentiation between stroke subtypes in nasal exudate seems possible since all the results obtained for the ischemic stroke are below the value of the first quartile in the hemorrhagic stroke. This impressive

finding indicates that nasal exudate could be employed as a new source of brain disease biomarkers. For example, metal concentrations have been analyzed in plasma and CSF of patients with Alzheimer's disease [16], but never before in nasal exudate, which is anatomically very closely related. In the specific case of the differential diagnosis of stroke this becomes very relevant because it would serve to establish a cut-off value, below which a stroke event would be safely diagnosed as ischemic and then it could be treated with thrombolytic agents in a faster way than is currently possible.

To establish such cut-off value that allows differentiation between both subtypes, an ROC curve was represented for both, saliva and nasal secretion samples. The cut-off value chosen implies that 90% of positive (hemorrhagic) and a 90% of negative (ischemic) values were correctly identified. Regarding the values of the AUC value and considering that a value of 0.5 has no any predictive value (similar to a random decision), the value for iron concentration in saliva samples cannot provide accurate results. However, the value of the AUC for nasal exudate samples was considered very good in which regards to its predictive value.

In summary, this article shows that nasal exudate, which can be sampled non-invasively, was employed for the first time to obtain information directly from brain. The analysis of saliva and nasal exudate samples shows that a simple nasal exudate analysis can give relevant information that it is very adequate for the differential diagnosis of stroke. This fluid: (i) can be obtained non-invasively (compared to venous blood or CSF that requires lumbar puncture). As it is an external secretion, it can be easily and rapidly obtained with swabs, even far from hospital settings by non-qualified personnel; (ii) can be extremely informative because it is closely (anatomically)-related to the brain; (iii) can contain low-diluted biomarkers (in the case of iron, which is in the order of  $\text{nmol mg}^{-1}$  of sample; considering samples of  $1 \text{ g mL}^{-1}$  of density this is in the  $\text{mmol L}^{-1}$  range) due to the short distance (from the brain) at which biomarkers are intercepted, (iv) the matrix is not as complex as blood, that is a more general "waste clearance collector". In any case there is no need to perform any complicate sample pretreatment and only dilution to enter the linear dynamic range of the analytical methodology is performed. All these advantages make nasal exudate a promising source of information, plenty of biomarkers that travel unnoticed from brain to cervical lymph nodes. Their easy interception at one of the brain exit doors paves the way to the study of this and other useful biomarkers of several neurological diseases (e.g. neurodegenerative disorders such as Alzheimer's and Parkinson's diseases,

where changes in iron homeostasis result in altered cellular distribution and accumulation [17]). In the specific case of iron, this is an easy-to-measure and promising biomarker, suitable to: (i) perform wider clinical studies that confirm this evidence and (ii) develop portable devices for decentralized diagnostics, useful also for remote settings and developing countries.

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