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Short Communication

In vivo electrochemically-assisted polymerization of conjugated functionalized terthiophenes inside the vascular system of a plant

Julie Pham [a], Amélie Forget [a], Nathalie Bridonneau [b], Giorgio Mattana [a], Eleni Stavrinidou [c], Samia Zrig [a], Benoit Piro [a], Vincent Noel [a,*]

[a] ITODYS, CNRS UMR 7086, Université de Paris Cité, F-75013 Paris, France
[b] ICMMO, CNRS UMR 8182, Université Paris Saclay, 91405 Orsay, France
[c] Laboratory of Organic Electronics, Department of Science and Technology, Linköping 8 University, 2 SE-601 74 Norrköping, Sweden

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ABSTRACT

We investigate the possibility of producing biofuel cell electrode materials in vivo by injecting the reagents directly into plant tissues. We first introduce model electroactive substances $Fe(CN)_6^{4-}$ and $Ru(NH_3)_6^{3+}$ into a *Nicotiana tabacum* leaf. In situ electrochemical measurements make it possible to trace the distribution of these substances. As well as mapping the vascular content, electrochemistry can be used to trigger reactions directly inside the plant. The injection of thiophene (T) and ethylenedioxythiophene (E)-based trimers (ETE) anchoring an Os(2,2′-bipyridine)$_2$(1-(3-aminopropyl)-imidazole)Cl Os-complex followed by the application of anodic polarization triggers a polymerization reaction in the region of the plant vascular system containing the monomer, showing that it is possible to generate electroactive organic macromolecules locally in vivo.

1. Introduction

Enzyme-based biofuel cells (EBFCs) have been considered for long-term internal implantation inside living bodies to supply energy to other functional devices such as biosensors [1]. EBFCs are based on enzymes able to catalytically oxidize sugars or reduce oxygen (e.g., glucose oxidase and bilirubin oxidase, respectively). From the first work of Yahiro et al. [2] to the most recently published results [3], significant progress has been made in the field of in vivo EBFC. However, to date, very few examples of EBFC focus on the plant world [4–8]. Mano et al. first reported the ability of a membraneless micro-EBFC to harvest energy from fruits [5–6] and from cacti [7]. However, there is a large set of plants not yet addressed by this technology, which offer interesting properties, e.g. a fast growth rate, low sensitivity to drought, a long lifetime and large size. This last property is of particular interest for current intensity improvement as larger plants are theoretically able to support large area current collectors. However, plants are composed of a vascular system, and electrode insertion into plant vascular tissue only engages a small contact area between the enzyme-modified electrode and the contents of the xylem and phloem; not to mention the injuries induced by insertion of the electrodes.

Inspired by the concept of "Living Plants as Technology" [8–10] and by the seminal work of Stavrinidou et al. [11–12], we propose to flip the paradigm of *in planta* biofuel cell electrode fabrication. The approach consists in introducing reactants into the vascular system and forming the biocathode and the bioanode in situ to catalyse and collect charge from the plant content. Given the accessibility of their vascular system, leaves are good candidates to support such functionality. The biocompatibility and versatility of thiophene-based trimers or terthiophenes [13–14] make them suitable precursors for in vivo formation of conductive polymers able to collect and transport charge arising from enzymatic reactions involving glucose and oxygen.

To achieve efficient collection of charge, several issues must first be addressed: (i) design and synthesis of redox mediators including a catalytic center for enzyme regeneration anchored to a π-conjugated molecule capable of electropolymerization into a conductive material (for charge transport); (ii) an analytical tool to probe or even to map the reactants in situ and (iii) a method of triggering local polymerization. Previous investigations of the molecular content of plants (e.g. glucose distribution [15], abscisic acid [16], heavy metals [17]) have conventionally involved the use of spectroscopic methods. In parallel, electrical-signal-based devices have been developed [7,18–20]. Reverse iontophoresis has been used to extract the vascular content of plants and analyze it using electrochemical methods [18] or HPLC [19]. Another

* Corresponding author.
E-mail addresses: vincent.noel@univ-paris-diderot.fr, vincent.noel@u-paris.fr (V. Noel).

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interesting approach is based on organic electrochemical transistors (OECT) used to monitor the sap electrolyte concentration in a growing tomato plant [20] and, more recently, to assay sugar content in situ and in real time in a poplar tree [21]. To date, no approach aimed at locally inducing chemical reactions has been proposed.

In this paper, we propose to study the embedding of biofuel cell electrode materials directly in the vascular system of a plant's leaves. Using model redox molecules, we first demonstrate the in vivo probing of electroactive materials in the vascular system of the plant using a three-electrode electrochemical setup. Then, ethylenedioxythiophene (E)-based trimers (ETE) anchoring an Os(2,2′-bipyridine)2(1-(3-aminopropyl)-imidazole)Cl Os-complex (ETE-OS) are introduced into the vascular system of the plant and electropolymerization is achieved in vivo.

2. Experimental

2.1. General methods

2.1.1. Solution uptake

For solution uptake via immersion, a leaf was cut from the tobacco plant and the petiole dipped in the solution of interest. In the case of uptake via agro-infiltration and injection, the leaf was left on the plant for administration of the solution of interest. Agro-infiltration was carried out by lightly puncturing the abaxial side of leaf, then forcing approximately 100 μL of solution at the puncture site using a disposable syringe. Injection was carried out using a Hamilton syringe which was inserted in the adaxial side of the petiole and approximately 25 μL of solution was injected.

2.1.2. In vivo electrochemical measurements

In vivo electrochemical measurements were performed with a 500 μm Au wire working electrode (WE) embedded in a glass tube (total electrode diameter ca. 700 μm), a 200 μm diameter Pt wire and a 500 μm diameter Ag/AgCl (homemade) wire as counter (CE) and reference (RE) electrodes, respectively. For the experiments with ETE-Os, a solution of ETE-Os and GOx was prepared and mixed with a glucose solution just before injection. The three-electrode system was then set up and cyclic voltammetry measurements initiated as quickly as possible. Only 30 s elapsed between the mixing of the two solutions and the CV measurements.

Cyclic voltammograms were acquired using an Autolab 8348 operated by Nova 2.0 software and processed using OriginPro. All the voltammograms were obtained at a scan rate of 100 $mV.s^{-1}$.

2.2. Materials and methods

Solvents were purchased from Aldrich and dried when necessary according to standard procedures. Reagents were used as received. 2,5-dibromo-3-(2-hydroxyethyl)thiophene 1, EDOT-2-boronic acid pinacol ester and [Os(2,2′-bipyridine)$_2$Cl$_2$] were synthesized using procedures reported previously [13,22–23]. Flash column chromatography (FCC) purification was performed using an Isolera One flash chromatography instrument (Biotage, Uppsala, Sweden).

2.3. Synthesis of [Os(2,2′-bipyridine)(1-(3-aminopropyl)-imidazole ETE)Cl] ETE-Os

Synthesis of 2,5-dibromo-3-(ethyl-2-tosylate)thiophene 2: 4-Toluenesulfonyl chloride (1 g, 5.25 mmol, see Fig. 1) was added to a solution of 2,5-dibromo-3-(2-hydroxyethyl)thiophene) **1** (1 g, 3.5 mmol) and pyridine (0.6 mL) in acetonitrile (40 mL) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C then at room temperature overnight. 40 mL of CH_2Cl_2 was added to the reaction mixture and washed with H_2O (40 mL), HCl (1 M, 40 mL) and saturated $NaHCO_3$ solution (40 mL). The organic layer was dried over $MgSO_4$ and evaporated. The crude compound was purified with FCC (KP-Sil SNAP 10 g, with CH_2Cl_2 as eluent) to yield **2** as a white powder (1.06 g, 68 %).

Synthesis of 2,5-bis[3,4-ethylenedioxythiophene]3-ethyl tosylate thiophene (ETE-OTs) 3: 2 (150 mg, 0.35 mmol), EDOT-2-boronic acid pinacol ester (190 mg, 0.71 mmol), PEPPSI- IPr Pd catalyst (14 mg, 21 μmol) and KF (135 mg, 2.33 mmol) were placed in a Schlenk tube and purged with argon. A degassed solution of THF/H_2O (10:2, 18 mL) was added under a flow of argon and the yellow solution was stirred under reflux (85 °C) for 24 h, during which it was observed to darken. The solution was then cooled to RT and partitioned between DCM and water. The DCM layer was concentrated, diluted in EtOAc and the organic layer washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The yellow crude product was loaded onto silica and purified by FCC (KP-Sil SNAP 10 g, 0 to 50% CH_2Cl_2:cyclohexane) to yield **3** as a yellow foam (136 mg, 68%).

Synthesis of 2,5-bis[3,4-ethylenedioxythiophene]-3-ethyl-N-aminopropylimidazole thiophene 4: 3 (70 mg, 0.125 mmol) and 1-(3-aminopropyl)-imidazole (78 mg, 0.623 mmol) were dissolved in CH_3CN and refluxed overnight. The solvent was removed in vacuo and the crude product was purified by FCC. (KP-Sil SNAP 10 g, Biotage, 8%

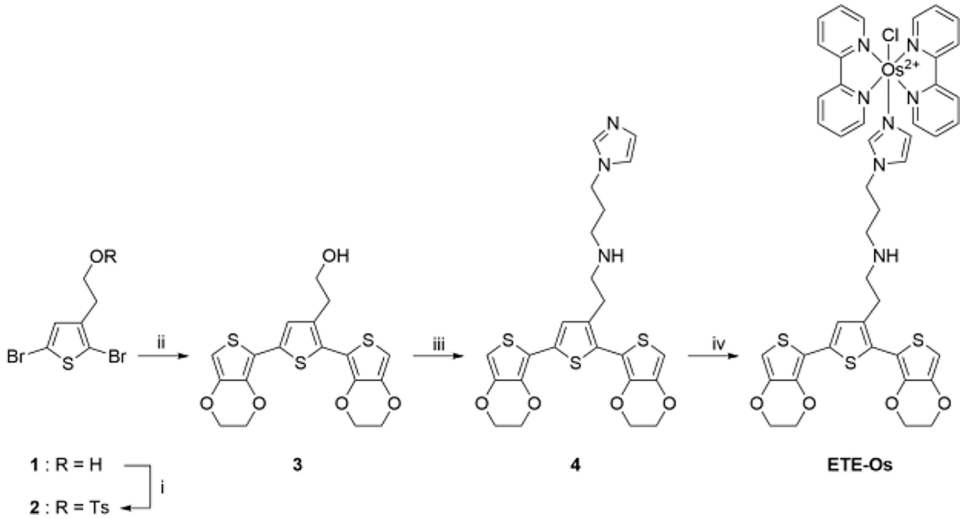

Fig.1. Synthesis of [Os(2,2′-bipyridine)(1-(3-aminopropyl)-imidazole ETE)Cl], **ETE-Os.** (i):TsCl, pyridine, CH_3CN, (68%); (ii) EDOT-2-boronic acid pinacol ester, PEPPSI-IPr Pd catalyst, KF, THF/H_2O (68%); (iii) 1-(3-aminopropyl)-imidazole, CH_3CN (62%); (iv) [Os(2,2′-bipyridine)$_2$Cl$_2$], ethylene glycol, microwave 180 °C.

MeOH: CH_2Cl_2) to yield **4** as a yellow oil, (39.9 mg, 62%).

Synthesis of [(Os(2,2′-bipyridine)$_2$(1-(3-aminopropyl)-imidazole ETE)Cl] ETE-Os: [Os(2,2′-bipyridine)$_2$Cl$_2$] (14 mg, 0.024 mmol) and **4** (26 mg, 0.048 mmol) were dissolved in ethylene glycol (2 mL) and microwaved at 180 °C for 10 min. The reaction mixture was diluted in ethanol and precipitated in cold Et_2O, then filtered using a PTFE membrane to yield **ETE-Os** as a black solid. ESI-MS/MS: calcd. for [M + H]+: m/z = 1053.7177; found: 1054.16647. Note that RMN data are available for compounds 1–4 and ETE-Os on request to the corresponding author.

3. Results and discussion

3.1. Investigation using model redox molecules

The simplest uptake method is the immersion of a cut petiole into a solution and the use of natural capillary motion in the plant vasculature. Agro-infiltration consists in actively administering exogenous solutions to plants without damage. The solution enters the leaf mesophyll area via the stomata, either by vacuum infiltration or by applying a positive pressure. Finally, injection to the plant vasculature, i.e. direct introduction of the liquid into the vascular system of a leaf using a microneedle, is another method which drives the uptake of a liquid by a living leaf. To assess the uptake techniques, we treated *Nicotiana tabacum* leaves with 1 mM solution of rhodamine. This colorimetric probe was agro-infiltrated at the middle of the leaf, on its abaxial side, or injected close to the petiole (adaxial). After 3 h, agro-infiltration led to a full distribution of the dye inside the leaf mesophyll. Immersion led to full colouration of the leaf xylem network by rhodamine. In the case of injection, the solution was locally distributed along the vasculature on both sides of the injection point (up to several cm, data not shown).

Using a 1 mM $K_3Fe(CN)_6$ uptake solution, we then evaluated the possibility of electrochemically detecting the molecular compounds in situ. The electrochemical cell consisted of three wires directly inserted into the vascular system of either a cut leaf or a leaf still on a living plant (see Fig. 2).

Cyclic voltammograms were acquired three hours after the uptake process had started. The electrochemical signature of the ferri/ferrocyanide reversible redox reaction was clearly seen for the three uptake modes investigated (see Fig. 3a,b). Agro-infiltration led to peak current intensities of ca. 1 μA while current intensities 4-fold and 20-fold higher were obtained for the injection and the immersion uptake modes, respectively. These experiments show that the ferri/ferrocyanide species diffuses poorly within the vascular system after agro-infiltration, making this administration method less pertinent to our study.

We further investigated the use of electrochemistry to probe the vascular content using the immersion uptake method. The content was investigated at various distances from the uptake point of a 1 mM $Fe(CN)_6^{3-}$ or $Ru(NH_3)_6^{3+}$ solution (see Fig. 3c). Both species were transported far from the uptake point, as shown by the strong redox signal recorded at a distances up to 16 cm. Note that current intensities must be handled carefully as the precise number of electrochemically connected plant veins may vary between experiments. Nevertheless, $Ru(NH_3)_6^{3+}$ seemed to be better transported, as shown by the higher current intensity recorded regardless of the distance from the uptake point. The rate of upward transport in the xylem is directly correlated to leaf transpiration. It is likely that transpiration is increased by $Ru(NH_3)_6^{3+}$, leading to a faster rise of this compound compared to $Fe(CN)_6^{4-}$.

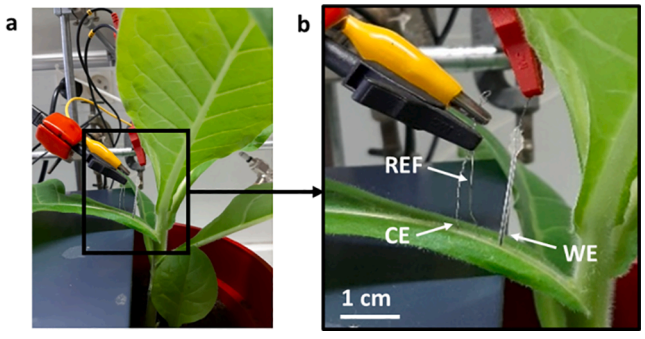

Fig. 2. (a) Electrochemical cell used for in vivo measurements; (b) enlarged picture of the cell.

The electrochemical measurement of the local vascular content thus makes it possible to map the distribution of redox substances in the plant. To investigate the transport rate of the redox probe inside the vascular system, the current was measured at different times after the leaf was immersed in a solution of $Fe(CN)_6^{4-}$ (see inset of Fig. 3a). The current remains very low and increases sharply after one hour of immersion to reach a stationary value. These results show that the proposed electrochemical method makes it possible to carry out measurements as a function of time with an electrode left in contact with the vascular content of a leaf.

3.2. Polymerisation within the plant's vascular system

We investigated the possibility of electrochemically triggering polymerisation reactions involving thiophene trimers previously taken up by the plant. We synthesized and injected ethylenedioxythiophene- and thiophene-based trimers anchoring either a redox Osmium complex (ETE-Os) (see experimental section, Fig. 1) or a sulfonate group (ETE-S) into a living leaf. We performed an electrochemical characterization of the content using the electrochemical setup described above. Irreversible anodic peaks between 0.6 and 0.8 V for ETE-S and ETE-Os, respectively, are characteristic of the oxidation of the ETE moieties (data not shown). The $Os^{III/II}$ reversible peak is observed in the case of ETE-Os injection with anodic and cathodic peak potentials at 0.15 and 0.05 V, respectively. After 2 to 3 h, a light black coloration appeared spontaneously inside the vascular system containing ETE-S, while the leaf containing ETE-Os showed no colour change. Dufill et al. links the colour change to the in situ polymerization of ETE-S under the activity of endogenous enzymes (pyruvates) [24]. The reason for the lack of ETE-Os reactivity is not clear, but it could be due to the in situ concentration of ETE-Os being too low, and/or insufficient enzymatic activity to trigger polymerization.

Our results suggest that polymerization is a combination of electropolymerization and enzymatic polymerization. To increase the rate of enzymatic polymerization of ETE-Os, several tests were carried out (1 mM H_2O_2, GOx, 30 mM glucose, 10 mM NaN_3, data not shown) and only the GOx/glucose mixture gave interesting results. GOx activity generates H_2O_2 locally, which is the endogenous pyruvate co-substrate. While pure H_2O_2 injection does not lead to polymerization, the in vivo production of H_2O_2 increases the polymerization rate, probably by increasing the activity of endogenous enzymes.

When the vascular system was investigated at the same location immediately after glucose/GOx injection, the ETE oxidation peak decreases continuously whereas the Os signal increases (see Fig. 4c) from the first to the fifth scan and remains stable from the 5th to the 10th scan. Simultaneously, a strong colour change appears in the vein (see Fig. 4b), indicating that polymerization of ETE-Os occurs under these conditions within the very short timeframe of the cyclic voltammetry experiment. No change is observed without glucose and GOx (see Fig. 4a). On the one hand, the colour of the observed vascular system changes, which is characteristic of the π-conjugation extension of the ETE segment [17]. On the other hand, π-conjugation extension of the ETE constituent should give rise to an electrochemical signal relative to its doping/dedoping. Unravelling the polymerization mechanism would shed light on this apparent contradiction. This requires additional development, in particular methods for extracting and analyzing the vascular content a posteriori, for example using reverse iontophoresis [18–19]. Nevertheless, some hypotheses can be outlined based on these

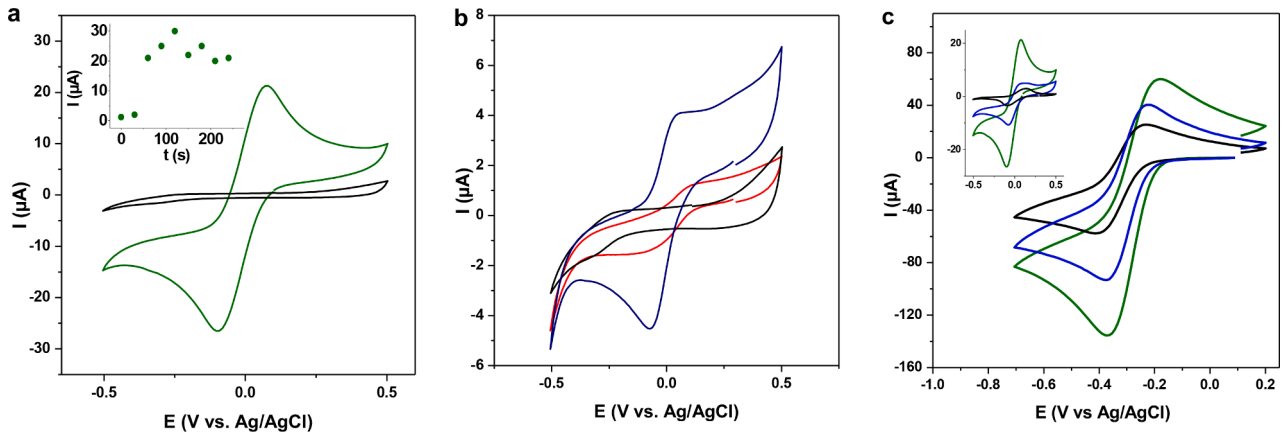

Fig 3. CVs acquired in a plant leaf treated with 30 mM $K_3Fe(CN)_6$: (a) (green) through immersion (inset: current at 0 V as a function of time of immersion in the uptake solution); (b) (blue) injection of 25 µL of solution and (red) agro-infiltration of 100 µL of solution; CV in untreated leaves are shown in black. (c) CVs acquired in the plant after 3 h immersion in 30 mM $Ru(NH_3)_6^{3+}$ or (inset) $Fe(CN)_6^{3-}$ solution, at various distances from the uptake point: 2 cm (green), 12.5 cm (blue), and 16 cm (dark). Scan rate v = 100 $mV.s^{-1}$.

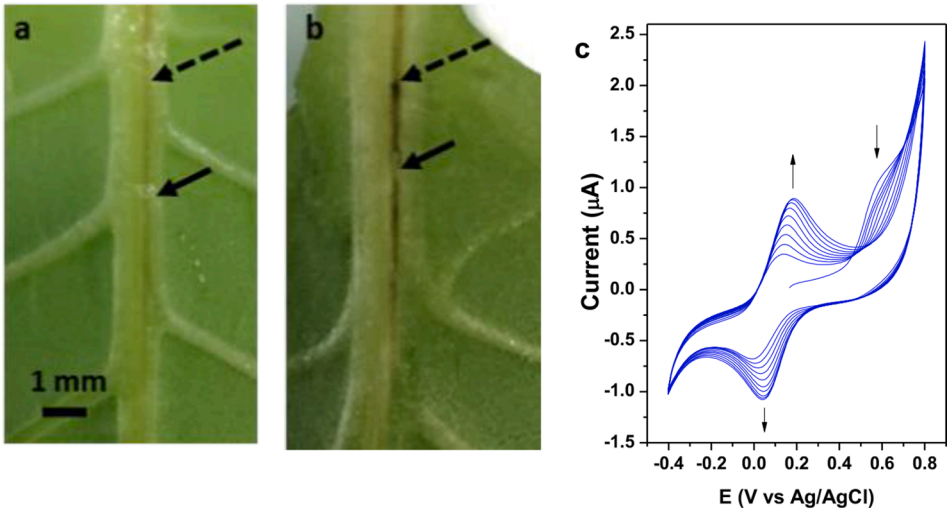

Fig. 4. : Pictures of a leaf after 10 scans at v = 100 $mV.s^{-1}$ into which (a) ETE-Os and (b) ETE-Os + glucose 30 mM + GOx [5 mg/mL] were injected; the solid arrows represent the injection position; the dashed arrows represent the working electrode position. (c) CVs acquired in vivo in a leaf containing ETE-Os + glucose 30 mM + GOx [5 mg/mL].

results. Indeed, polymerization of ETE-Os might lead to the formation of short oligomers that precipitate on the walls of the vascular system. As the electrode punctures the vascular system, some of the oligomers on the walls are in direct contact with the electrode and are consequently connected to it. The resulting material behaves here more like a redox polymer than a conjugated polymer (charge hopping through metallic centers instead of charge transport through a conjugated backbone). According to the stable Os signal finally obtained, some of the Os-based redox oligomers can be addressed in vivo.

A study of the effect of the polymerization process on leaf survival can be achieved by using interfacing systems that have shown their harmlessness for several days (e.g. [21]) and minimal concentrations of reagents. After these improvements have been set up, we plan to apply this methodology to in vivo polymerization and electrochemical characterization of a polymer matrix including an electrochemical function of interest, for example an enzymatic mediator.

4. Conclusion

In vivo electrochemical experiments in plants are complex due to the high variability of the contents of the vascular system (hydrogen peroxide, enzymes…) which depend on many parameters, including illumination, humidity and stress. However, the proposed electrochemical setup allows for an accurate probing of the content of a plant's vascular system. We have shown that the spatial and temporal distribution of electroactive molecules can be determined. In addition, exogenous molecules, such as polymer precursors, can be locally injected into the vascular system and switched on electrochemically, leading to a polymerization process in the vascular region containing the injected solution. On the basis of the proposed methodology, ETE-Os molecules showing different standard potentials could be injected and polymerized at a given location inside the plant vascular system as in vivo supported electrocatalysts.

CRediT authorship contribution statement

Julie Pham: Investigation. **Amélie Forget:** Investigation. **Nathalie Bridonneau:** Investigation. **Giorgio Mattana:** Methodology. **Eleni Stavrinidou:** Validation, Funding acquisition. **Samia Zrig:** Methodology. **Benoit Piro:** Project administration, Methodology. **Vincent Noel:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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