

# Proof of nicotine transfer to rat pups through maternal breast feeding to evaluate the neurobehavioral consequences of nicotine exposure

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## Abstract

This study investigates the transfer of nicotine from lactating dams to their offspring through breast milk, in the frame of a research focused to ascertain toxicological and neuro-behavioural effects on pups as consequence of either unavoidable ("yoked & forced") or voluntary ("freely-chosen") maternal nicotine exposure. To this aim, plasmatic concentrations of nicotine and cotinine were determined by LC-MS/MS in Wistar rat pups whose mothers were orally administered with nicotine during lactation. Mothers were divided into a voluntary drinking group, an unavoidable consumption group, and controls. The limits of detection and quantification of the LC-MS/MS method were 0.20 and 0.65 ng/mL, respectively. Within-laboratory reproducibility (CV%) was <12%, with recovery of 86.2-118.8%. Results showed the presence of nicotine in 67% of samples from freely-chosen consumption group ( $1.30 \pm 0.31$  ng/mL) and in 60% of samples from yoked-consumption group ( $1.19 \pm 0.62$  ng/mL); cotinine was found in all the samples from freely-chosen ( $1.92 \pm 0.77$  ng/mL) and yoked-consumption groups ( $1.43 \pm 0.30$  ng/mL). Data provide an evidence-based support to maternal/offspring nicotine transfer as function of different ways of oral exposure.

## Key words

- maternal nicotine exposure
- free-choice drinking
- forced/yoked intake
- nicotine
- cotinine

## INTRODUCTION

Tobacco smoking entails a two-faced risk for human health: the risk of organic disease due to the exposure to toxic and carcinogenic chemicals, produced by tobacco burning, and the risk of neuro-behavioral effects (addictive risk), due to the exposure to nicotine that is a strong psychoactive compound able to induce dependence. In particular, tobacco when burning produces more than 7000 chemicals, of which hundreds are toxic and about 70 are carcinogens [1]. Nicotine is a tertiary amine composed of a pyridine and a pyrrolidine ring, and is the primary alkaloid found in Solanaceae and in particular in the plant *Nicotiana tabacum*. The leaves have a high nicotine content (accounting for 96-98% of the total alkaloid content) [2] and undergo a complex production process, needed to obtain products ready for human consumption by smoking, chewing, and snuffing. In tobacco, nicotine is largely present as the levorotary (S)- or (-)-isomer (99.4 - 99.7% of whole nicotine content) which is the most physiologically active isomer [3].

Nicotine belongs to the group of the cholinergic agonists and binds the acetylcholine receptors at ganglia, at neuromuscular junctions and in the brain [4]. Nicotine from the tobacco smoke is rapidly adsorbed into the bloodstream and metabolized in the liver. Due to its lipophilic character, nicotine penetrates easily through the blood-brain-barrier reaching the brain in just seconds (10-20 s) [3] after inhalation, and therefore producing central effects. Nicotine is able to influence cognitive functions, emotions and the reward processing, all of which play a key role in addiction [5-7]. Acute toxicity of nicotine, expressed as median lethal dose (LD50), is 0.5-1.0 mg/kg of body weight for humans [8]. Intoxication may occur from dermal absorption or accidental ingestion, especially by children for which the LD50 is only 0.1 mg/kg [9]. Accidental nicotine poisoning may occur by cigarette but also by nicotine gum or by refill liquids for electronic cigarettes [10-11].

As regards the teratogenic risk of nicotine exposure, the Food and Drug Administration (FDA) classifies

nicotine as “reproductive or developmental toxicant” [12]. Nicotine crosses the placenta and hence even small amounts could be toxic during pregnancy. Whereas the dangerous effects of smoking during pregnancy have been extensively depicted, it is not clear yet if and how nicotine affects neonatal development when exposure occurs during lactation. During pregnancy, maternal smoking is related to cognitive deficits in the offspring and to behavioural abnormalities as hyperactivity and attention deficit disorders [13].

In our perspective, it is often neglected that, further to smoke breathing by the baby, nicotine is secreted into the breast milk [14]. As stated before, nicotine is a lipophilic compound, so the likelihood of passing from the mother to the offspring through breast milk could be high. Therefore, major concerns should arise when mothers, which might have stopped smoking when realizing to be pregnant, do start smoking again during the crucial period of lactation, as well for mothers who continue and never quit smoking. On the other hand, it is also useful to obtain information about the risks and possible consequences of a kind of uncontrollable, unavoidable intake, often used in pharmacological literature: to this purpose, we included a group with a fully yoked nicotine exposure. In our study, lactating offspring were investigated after postnatal nicotine exposure offered to their rat dams. With the aim to assess nicotine transfer through milking, maternal exposure was obtained in two ways: (1) freely controllable drinking by own choice, with a relatively higher dosage of nicotine in water, and (2) forced and unavoidable intake, with a lower and yoked dosage of nicotine in tap water.

To these aims, it is required to provide a reliable quantification of nicotine actually detectable in the biological samples; as a biomarker, nicotine is highly specific even if not suitable for a routine clinical use, because of nicotine's short half-life [3]. Nevertheless, in most mammals about 70–80% of nicotine is extensively metabolized to the 5'-hydroxy-nicotine (cotinine), while other metabolites are also produced [3]. Cotinine is present in the blood in much higher concentrations than those of nicotine and it has a much longer plasmatic half-life, since the mean half-life is 16 h for cotinine with respect to just 2 h for nicotine [3]. For these reasons, cotinine is considered a highly specific and sensitive biochemical marker of nicotine exposure and it is widely used in clinical practice and for animal experimentation studies. However, cotinine in the pups' body is, in itself, a marker of maternal exposure, but not necessarily a proof of nicotine transfer nor a marker for actual offspring exposure. Indeed, detection only of this metabolite in the pups' body cannot dismiss the possibility that cotinine alone is transferred through the milk, and that nicotine never entered the pups' body.

To date, many analytical methods have been published to measure nicotine and cotinine in different biological samples such as serum/plasma, urine, saliva, hair, meconium. The most used analytical methods are Radio Immuno Assay (RIA) [15], Enzyme Immuno Assay (EIA) [16–18], Gas-Chromatography (GC) [17, 19, 20], and Liquid-Chromatography (LC) [17, 21–

27]. In particular, among LC techniques, the liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides a rapid, sensitive and selective tool for the determination of nicotine and all its metabolites [21–27]. The main focus of the present study was to investigate the actual passage of nicotine from dams to their offspring through breast milk. This is a crucial step, in order to correctly evaluate the neurobehavioral consequences of “direct” nicotine exposure, if confirmed, rather than “mere” exposure to maternal cotinine, if not so, within vulnerable, developing brains. In particular, we have been able to detect levels of nicotine, and not only of its major metabolite, cotinine.

Nicotine and cotinine were determined in the offspring plasma, which was collected with extreme care: the time of sacrifice was planned in a window between 40 and 50 min after a breastfeeding bout lasting at least 18 min consecutively. Pups' samples were indeed taken 45 min following the end of a lactation episode. A high sensitive and specific LC-MS/MS procedure was specifically set up for identification and quantification of nicotine and cotinine in the reduced volumes of plasma that can be obtained from these small animals. The analytical procedure and its validation are described in detail in the present paper. Within the experimental groups submitted to this kind of nicotine exposure, only some of the animals were sacrificed for determining plasmatic nicotine/cotinine. The other siblings underwent behavioural tests to investigate the impact of nicotine exposure on social and emotional behaviour of the adolescent offspring: this part of the study was described in a recent paper by some of the present authors [28].

## MATERIALS AND METHODS

### *Ethical note*

All experimental procedures were formally approved by the Italian Ministry of Health (formally valid during years 2014–2016, licence to GL), and were conducted in close agreement with the European Communities Council Directive (2010/63/EU) and Italian Law (D.Lgs 26/14).

All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilise alternatives to *in-vivo* techniques, if available.

### *Sample collection for method development and validation*

Pooled blood samples from adult male Wistar rats spiked with standards solutions were used to perform preliminary method development and validation. Blood was collected from the trunk into vials with heparin to prevent clotting, then centrifuged at 2500 r/min for 20 min at 4 °C. Plasma was transferred into tubes and then stored at -80 °C until further processing.

### *Subjects and housing*

Twenty-four female and twelve male Wistar rats, originally purchased from Charles River (France) were bred in our laboratory. Two females were housed with one male in polycarbonate cages (48.0 x 26.5 cm, height 21.0 cm), with sawdust bedding and a metal top. After 4 days of mating, male rats were removed. Sixteen

females were actually pregnant and were housed individually starting from the 16th day of gestation. Dams were daily checked for delivery; births occurred within 5 days. On the day after birth, designated as PND 1, culling was performed to keep 5 male and 3 female pups per mother.

Dams and their litters were maintained under standard facility rearing conditions (cage cleaning once a week) in an air-conditioned room (temperature  $21 \pm 1$  °C, relative humidity  $60 \pm 10\%$ ) on a reversed 12 : 12 hours light : dark cycle (lights on from 18:45 to 6:45), with water and rodent food pellets (Altromin - Rieper Maintenance Diet for Rats and Mice purchased from A. Rieper S.p.A., VanDOies, Bolzano, Italy) being available ad libitum.

### Neonatal treatments and procedures

Dams were divided in two batches used to compose three experimental groups, according to the day of delivery (pups belonging to each batch were born within a range of 3 days): the first 6 dams to give birth (batch A) were given a choice between two identical bottles, containing either a 3.5 mg/L nicotine solution or tap water (freely choosing drinking group); other 5 dams who gave birth afterwards (batch B) were provided with two identical bottles, both with an equal nicotine solution (forced and yoked intake group). The concentration of nicotine for batch B has been calculated based on the actual intake of batch A. Thus, as calculated daily, each of the two bottles for batch B contained a concentration that was corresponding to half of daily nicotine intake, measured by the free-choice fluid consumption already shown by dams on batch A. The third group was composed of 5 dams who received two identical bottles, both with tap water (controls). To avoid a bias represented by potential preferences for either the right or the left side of the cage, the position of the nicotine bottle was daily reversed as previously reported [29-30]. Apart from the different solutions in the drinking bottles, environmental condition were the same among groups.

Treatments with nicotine and water bottles started at PND  $3 \pm 1$  and lasted until PND  $12 \pm 1$ . Lactating dams belonging to all groups drank comparable amounts of fluid ( $66.35 \pm 1.20$  mL); daily nicotine intake fluctuated over the 10 treatment days, ranging between 0.20 - 0.62 mg/kg ingested with  $29.74 \pm 2.56$  mL of fluid (average preference 45.9%), and 0.12 - 0.34 mg/kg ingested with all drunk fluid, for the "free-choice" drinking and the "forced/yoked" intake groups, respectively. "Actively choosing" mothers drank nicotine at their wish, whereas for "forced/yoked" dams were unavoidably obliged to drink the nicotine solution whenever they were thirsty. After PND  $12 \pm 1$ , all dams were returned to tap water.

Two male and one female pups per dam were decapitated for collecting blood from the trunk at PND  $12 \pm 1$  at specific timing (range: 40 to 50 min after a breastfeeding bout lasting at least 18 min consecutively). Blood samples were collected to obtain plasma, as described in previous 2.2 section, for subsequent quantitative determination of nicotine and cotinine. Remaining pups were submitted to behavioural tests

during adolescence, according to procedures published elsewhere [28].

### Chemicals and reagents

Nicotine hydrogen tartrate salt used for nicotine administration was purchased by Sigma-Aldrich (St. Louis, MO). Heparin solution for sample collection was obtained by 5000 U.I./mL Heparin Vister provided by Marvecs Pharma Services S.r.l. (Milan, Italy). Methanol, diethyl ether, ethyl acetate and acetonitrile used for sample preparation and LC-MS/MS analysis were HPLC or analytical grade and were purchased from Sigma-Aldrich. Water was purified by Synergy-UV-System (Millipore, Bedford, MA, USA). Ammonium bicarbonate, (-)-nicotine and (-)-cotinine were provided by Sigma-Aldrich. OASIS HLB SPE cartridges (3 mL, 60 mg) were supplied by Waters (Milford, MA, USA). ( $\pm$ )-nicotine-d4 (100  $\mu$ g/mL, 1 mL, 98 % D) was used as Internal Standard (IS) and was provided by Sigma-Aldrich (St. Louis, MO).

The nicotine solution for the free-choice consumption group (batch A) was freshly prepared by dissolving 10.0 mg of nicotine hydrogen tartrate salt in 1000 mL of tap water, to obtain a final nicotine concentration of 3.5 mg/L. The nicotine solution for the forced consumption group (batch B) was freshly prepared each day, by dissolving an appropriate amount of nicotine; this, was calculated on the basis of daily chosen nicotine intake of the free-choice consumption group, already shown for the corresponding post-natal day of exposure in batch A, and then halved (ranging from 0.26 mg to 1.44 mg, on average 1.15 mg). The 5% ammonia solution was prepared by diluting an 20% ammonia solution (Carlo Erba Reagents, Milan, Italy). The 20 mM ammonium bicarbonate solution was obtained by dissolving 1.58 g of ammonium bicarbonate in 1000 mL of purified water.

### Standards and matrix calibration curve for LC-MS/MS analysis

A 1 mg/mL stock solution of each standard (nicotine and cotinine) was prepared in methanol, and from these solutions, 10  $\mu$ g/mL dilutions were prepared. Stock solutions, regularly analysed at fixed times to assess their stability in the time, did not exhibit degradation of the analytes up to six month

A mixture of standards was then prepared to obtain working solutions at dilutions of 100, 50 and 10 ng/mL. IS working solution was prepared by serial dilutions to obtain a concentration of 98 ng/mL. Working solution, stored at 4 °C, were prepared daily and used to spike blank (i.e. without nicotine and cotinine) plasma to obtain the matrix-matched calibration. Calibration curve for determination of linearity and quantification was constructed by spiking 0.5-mL-aliquots of blank plasma with 25  $\mu$ L of 98 ng/mL IS (final concentration: 4.9 ng/mL) and with working solutions to obtain 5 concentration points: 0.65, 1.30, 2.55, 5.10 and 10.20 ng/mL for nicotine and 0.65, 1.31, 2.63, 5.25, and 10.50 ng/mL for cotinine. Three set of replicates of each calibration levels on three different days were used to determine linearity.

### Sample preparation

Each 0.5-mL-aliquot of offspring plasma was spiked with a methanolic solution containing known amount of IS (25  $\mu$ L, 98 ng/mL) and then was added to 0.5 mL of 5% ammonia solution. The mixture was vortex-mixed and then loaded on the OASIS HLB SPE cartridge which was previously conditioned with 3 mL of methanol and 3 mL of purified water. After the washing step with 3 mL of purified water, the analytes were eluted with 2 mL of ethyl acetate/diethyl ether 2: 1 (v/v). The extract was then evaporated under nitrogen stream and the dry residue dissolved in 100  $\mu$ L of a mixture of methanol and 20 mM ammonium bicarbonate (9 : 1, v/v) for LC-MS/MS analysis.

### LC-MS/MS analysis

Analyses were carried out using a LC system Perkin Elmer Series 200 Micro Pump equipped with a PE Series 200 auto sampler (Perkin Elmer, USA). The chromatographic separations were obtained under gradient conditions at room temperature (25 °C) using a reverse phase HPLC column Gemini-NX C18 110A (150 x 4.60 mm, I.D. 5  $\mu$ m) (Phenomenex, USA) with a Gemini-NX C18 guard column (4 mm x 3 mm I.D.) (Phenomenex, USA). The mobile phase was composed of ammonium bicarbonate 20 mM (mobile phase A) and acetonitrile (mobile phase B) and the flow rate was 0.8 mL/min. The gradient profile began at 90% A and changed to 5% in 9 min, then returning to 90% A in 0.3 min and held for 3 min.

The API 3000 triple quadrupole mass spectrometer (AB Sciex, Canada) was equipped with an Electro Spray Ionization (ESI) source, and was set in positive ionization mode with a source temperature of 450 °C and an ionspray voltage of 5500 V; ultra-pure nitrogen was used as curtain and collision gas, and ultra-pure air was used as nebulizer and auxiliary gas. The full identification of the analytes was achieved according to the criteria of the Commission Decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results [31] and the peak areas of the analytes were computed using the software Analyst version 1.4 from AB Sciex.

According to the Decision 2002/657/EC, the 4 points required for identification by LC-MS/MS technique ("identification points") were yielded using the MRM (Multiple Reaction Monitoring) mode with one precursor ion and two product ions (two transitions). *Table 1*

reports the optimized parameters of nicotine, cotinine and IS and *Figure 1* (a and b) shows Positive-ESI MS/MS spectra and proposed fragmentation pathways for nicotine and cotinine) [18, 21, 22, 24-26]. *Figure 2* shows a representative extract ion chromatograms for nicotine, cotinine, and IS in a spiked plasma sample

### Statistical analysis

Analytical data, expressed as mean  $\pm$  standard deviation, were analysed using repeated-measures analysis of variance (ANOVA) to compare plasmatic concentration of both nicotine and cotinine found in the free-choice drinking group versus the yoked-intake unavoidable consumption one.

## RESULTS

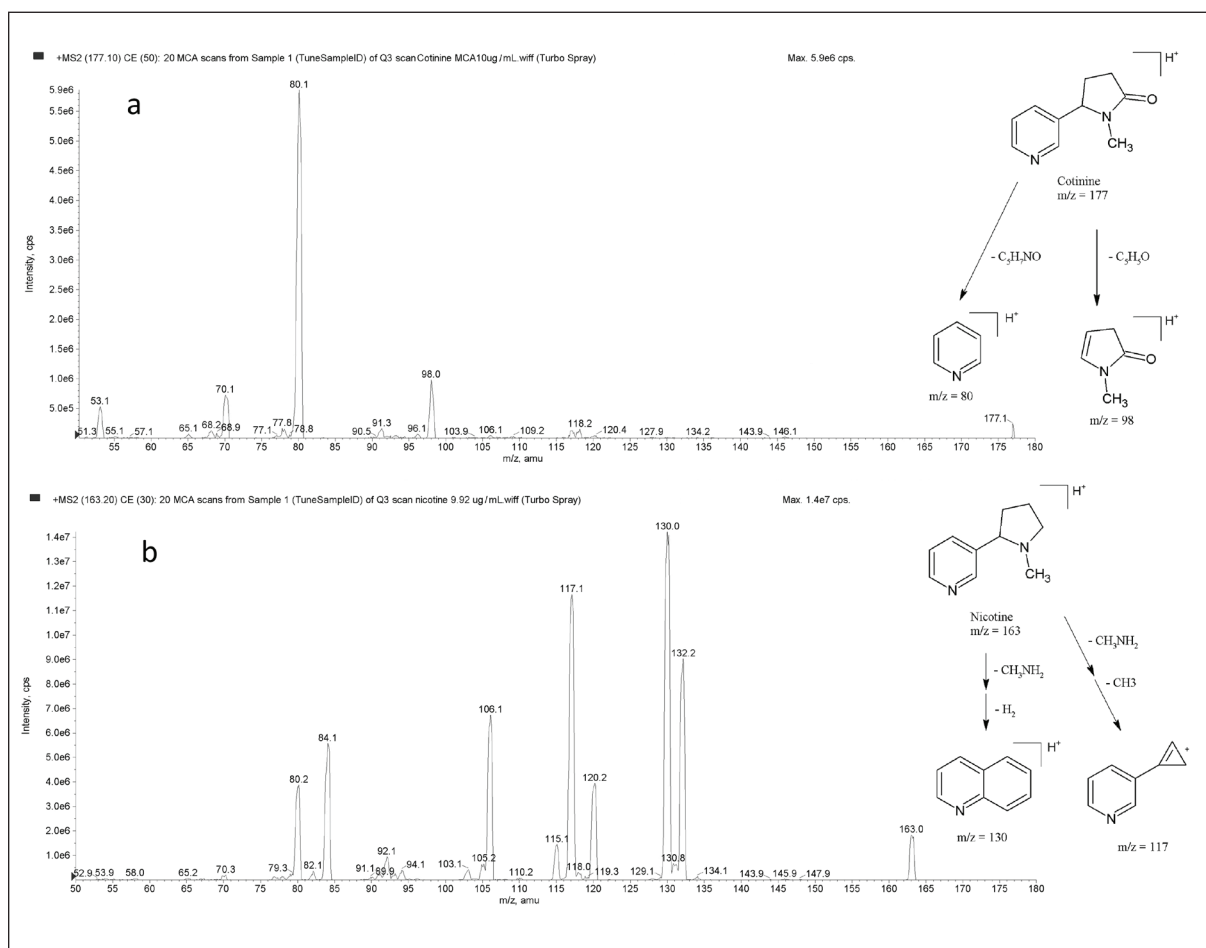
### Validation of LC-MS/MS analytical procedure

The linearity was assessed by the matrix-matched calibration curve, built with five calibration levels for nicotine and cotinine in the selected concentration intervals described above. The linear regression analysis showed a correlation coefficient greater than 0.998. As regards the Limit Of Detection (LOD) and the Limit Of Quantification (LOQ) (i.e. the lowest concentrations of the analyte that can be reliably detected and measured, respectively), in terms of signal-to-noise ratios  $\geq 3$  and  $\geq 10$ , they resulted respectively equal to 0.20 ng/mL and 0.65 ng/mL for both nicotine and cotinine. Analytical selectivity and specificity of methods were assessed directly onto the chromatograms obtained from the blank and from spiked plasma matrices. The occurrence of possible extra-peaks was tested by monitoring the two MRM transitions characteristic for each investigated compound onto the blank matrix chromatograms, in the retention time window expected for elution of the analyte. Repeatability expressed as intra-day coefficient of variation (CV%) was evaluated by analysing daily four replicates of samples at three concentration levels: 0.65 (corresponding to LOQ value), 2.55 and 10.20 ng/mL for nicotine; 0.65 (corresponding to LOQ value), 2.63 and 10.50 ng/mL for cotinine. At the LOQ values, the intra-day CV% resulted below 11% for nicotine and below 10% for cotinine. At the two higher concentration levels intra-day CV% ranged between 2.39% and 7.01% for nicotine, and between 2.09% and 6.37% for cotinine. To evaluate the within-laboratory reproducibility and recovery, the same series were analysed over three different days. The within-laboratory reproduc-

**Table 1**  
Precursor and most abundant product ions and their optimized parameters

| Analyte                      | Transition             | Collision Energy (eV) | Declustering Potential (V) | Retention time (min) |
|------------------------------|------------------------|-----------------------|----------------------------|----------------------|
| Nicotine                     | 163 > 130 <sup>a</sup> | 30                    | 40                         | 5.7                  |
|                              | 163 > 117              | 35                    | 40                         |                      |
| Cotinine                     | 177 > 80 <sup>a</sup>  | 35                    | 40                         | 4.3                  |
|                              | 177 > 98               | 30                    | 40                         |                      |
| Nicotine-d <sub>5</sub> (IS) | 167 > 134 <sup>a</sup> | 30                    | 40                         | 5.7                  |
|                              | 167 > 121              | 35                    | 40                         |                      |

<sup>a</sup> Most abundant product ion



**Figure 1**  
Positive-ESI MS/MS spectra and proposed fragmentation pathways for nicotine (a) and cotinine (b).

ibility expressed as inter-day reproducibility CV% was below 12.0% for both nicotine and cotinine at LOQ values and below 9.0% at other concentration levels within the matrix calibration interval (Table 2). Recovery percentage within calibration interval ranged from 86.2% to 118.8% for both the analytes.

### Biochemical markers of nicotine exposure through maternal milk

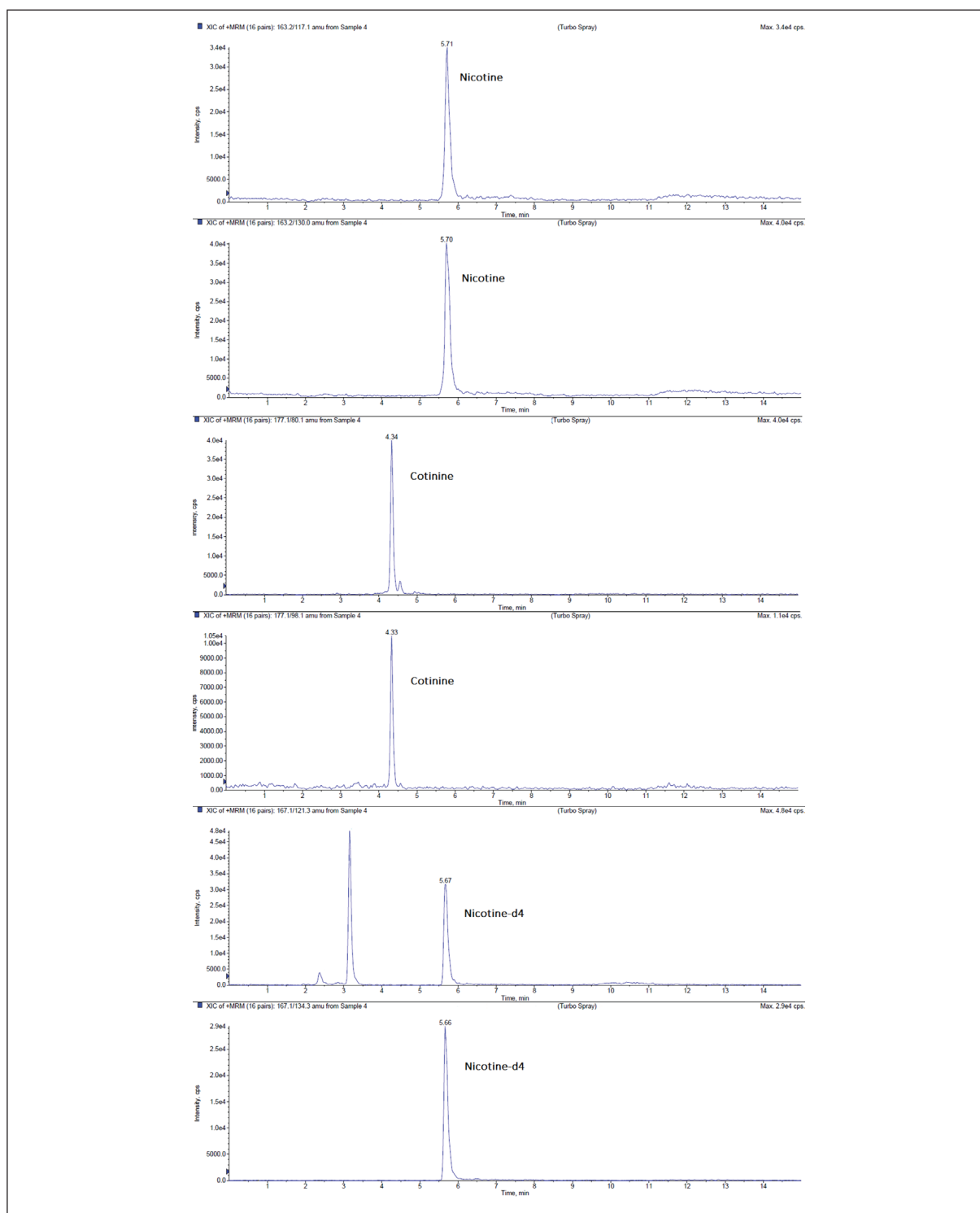
Biochemical quantification performed in the off-spring plasma confirmed the presence of nicotine in 67% of samples belonging to the free-choice drinking group ( $1.30 \pm 0.31$  ng/mL) and in 60% of samples belonging to the “yoked” intake group ( $1.19 \pm 0.62$  ng/mL). Cotinine was conversely determined in all (100%) of samples belonging to the free-choice drinking group ( $1.92 \pm 0.77$  ng/mL) and in all (100%) of samples belonging to the “yoked” intake group ( $1.43 \pm 0.33$  ng/mL). No traces of nicotine and cotinine were found in plasma of control rats, as expected. Analytical data were analysed using repeated-measures analysis of variance (ANOVA) to compare plasmatic concentration of both nicotine and cotinine found in the free-choice drinking group versus the “yoked” intake one. No significant differences were found between the two experimental groups ( $F(1,5) = 0.09$ ,  $P = 0.78$ , NS). These results

were subsequently considered to evaluate the results of the behavioural tests in the adolescent subjects.

### DISCUSSION

The main focus of the present study was to ascertain the nicotine transfer by maternal milk to lactating pups and, to this aim, a specific model of nicotine exposure and an appropriate LC-MS/MS procedure suitable for determining plasmatic nicotine and cotinine level of pups were set up. In particular, the study was focused on two maternal exposure scenarios, for investigating if the way by which dams assume nicotine orally could affect lactating offspring. The two experimental groups were composed from mothers exposed to either free-choice (i.e., freely choosing between bottles) or unavoidable (“forced / yoked”) oral consumption of nicotine (through drinking water). Furthermore, the nicotine exposure through milk in suckling pups was studied as one of the most “natural ways” of exposure to nicotine that may occur in a human baby.

Cotinine, that is usually detected alone in the off-spring, may be produced in the liver of dams and then transferred through milk. So, its detected presence does not necessarily imply nicotine transfer from mother with then production by the liver of pups. Only a reliable detection of the actual presence of nicotine in the



**Figure 2**  
Representative extract ion chromatograms for nicotine, cotinine, and nicotine d4 (IS) in a spiked plasma sample.

offspring plasma could give an evidence-based prove of nicotine transfer by maternal milk. It is evident that such a crucial information is a base on which to rely for evaluating related effects and long-term sequelae. So, it was crucial to provide the evidence and proof of nicotine transfer by an analytical procedure suitable for quantifying plasma nicotine and cotinine in the present

experimental conditions. For the preparative phase of our analysis, different solid phase extraction cartridges with different eluting solvents were tested and OASIS HLB SPE cartridges yielded the best recovery for both the two analytes, among the various tested procedures.

Plasma samples (taken 45 min after a bout of lactation, not with random timing as is usually the case)

**Table 2**

Calculated repeatability (intra-day) and within-laboratory reproducibility (inter-day) for the LC-MS/MS method for analysis of nicotine (NC) and cotinine (CT)

| Repeatability (intra-day)                     |         |      |      |       |                                               |      |      |      |                                                  |       |      |      |
|-----------------------------------------------|---------|------|------|-------|-----------------------------------------------|------|------|------|--------------------------------------------------|-------|------|------|
| I level:<br>0.65 ng/mL (NC, CT)               |         |      |      |       | II level:<br>2.55 ng/mL (NC), 2.63 ng/mL (CT) |      |      |      | III level:<br>10.20 ng/mL (NC), 10.50 ng/mL (CT) |       |      |      |
| Day 1                                         | Analyte | Mean | SD   | CV%   | Analyte                                       | Mean | SD   | CV%  | Analyte                                          | Mean  | SD   | CV%  |
|                                               | NC      | 0.60 | 0.06 | 10.52 | NC                                            | 2.50 | 0.18 | 7.01 | NC                                               | 9.48  | 0.23 | 2.39 |
|                                               | CT      | 0.52 | 0.04 | 8.14  | CT                                            | 3.18 | 0.19 | 5.92 | CT                                               | 11.10 | 0.32 | 2.85 |
| Day 2                                         | Analyte | Mean | SD   | CV%   | Analyte                                       | Mean | SD   | CV%  | Analyte                                          | Mean  | SD   | CV%  |
|                                               | NC      | 0.53 | 0.06 | 10.66 | NC                                            | 2.63 | 0.14 | 5.30 | NC                                               | 10.24 | 0.37 | 3.58 |
|                                               | CT      | 0.61 | 0.06 | 9.42  | CT                                            | 3.29 | 0.21 | 6.37 | CT                                               | 12.68 | 0.29 | 2.27 |
| Day 3                                         | Analyte | Mean | SD   | CV%   | Analyte                                       | Mean | SD   | CV%  | Analyte                                          | Mean  | SD   | CV%  |
|                                               | NC      | 0.55 | 0.06 | 10.38 | NC                                            | 2.53 | 0.14 | 5.62 | NC                                               | 9.88  | 0.26 | 2.61 |
|                                               | CT      | 0.63 | 0.05 | 7.89  | CT                                            | 2.87 | 0.17 | 6.01 | CT                                               | 11.96 | 0.25 | 2.09 |
| Within-laboratory reproducibility (inter-day) |         |      |      |       |                                               |      |      |      |                                                  |       |      |      |
| I level:<br>0.65 ng/mL (NC, CT)               |         |      |      |       | II level:<br>2.55 ng/mL (NC), 2.63 ng/mL (CT) |      |      |      | III level:<br>10.20 ng/mL (NC), 10.50 ng/mL (CT) |       |      |      |
|                                               | Analyte | Mean | SD   | CV%   | Analyte                                       | Mean | SD   | CV%  | Analyte                                          | Mean  | SD   | CV%  |
|                                               | NC      | 0.56 | 0.06 | 11.04 | NC                                            | 2.55 | 0.15 | 5.93 | NC                                               | 9.87  | 0.41 | 4.20 |
|                                               | CT      | 0.59 | 0.07 | 11.53 | CT                                            | 3.12 | 0.28 | 8.93 | CT                                               | 12.06 | 0.69 | 5.74 |

SD: standard deviation; CV: coefficient of variation.

underwent a previous clean-up/concentration step by Solid-Phase Extraction (SPE) cartridges. Subsequently, the obtained extracts were reconstituted with an appropriate solvent and injected into the LC-MS/MS system. The LC-MS/MS technique coupled to SPE clean-up / concentration of samples allowed the use of small amount of samples without loss of analytical sensitivity and specificity. Evaluated performance characteristics of the developed analytical procedure were: linearity, selectivity/specificity, limit of detection (LOD), limit of quantification (LOQ), repeatability (intra-day CV%), within-laboratory reproducibility (inter-day CV%), and recovery. Obtained results from nicotine exposed rats were then compared with those from non-exposed rats, belonging to the control group.

The criteria for molecular identification are those of liquid chromatography coupled to tandem mass spectrometry and it was performed by checking the matching of the relative retention time observed for the analyte peaks in samples compared to methanolic standard analytes, with a tolerance of  $\pm 2.5\%$ . Moreover, the two transitions from the analyte molecular peak were monitored with a signal-to-noise ratio greater than 3. All ion ratios of samples were within the recommended tolerances as required by Decision 2002/657/EC [31] when compared with standards. The within-laboratory reproducibility (inter-day CV%) was below 12.0% for both nicotine and cotinine at LOQ values, and below 9.0% at other concentration levels within the matrix calibration interval. The recovery ranged from 86.2% to 118.8% for both the analytes. These calculated performance characteristics showed that the procedure is suitable for clinical studies and even for animal toxicology experimentations conducted on rodents or other similarly

small animals, whose available plasma volumes are generally just few microliters.

The analytical procedure herein presented was applied to investigate whether nicotine passes from dams to their offspring through breast milk and to quantify plasmatic nicotine and its major metabolite cotinine in plasma from Wistar rat pups whose mothers were orally administered with nicotine solutions during ten days of the lactation period. Analytical results confirmed that this passage exists and that nicotine and cotinine are detectable in the plasma of the offspring after a bout of lactation.

Comparison between the two nicotine treated groups showed no reliable nor significant differences in nicotine and cotinine concentrations. As for this finding, some considerations may be put forward. It seems that, during transfer, some kind of rate-limiting ceiling was reached. Indeed, dams under a free-choice condition voluntarily ingested a remarkable quantity of nicotine, available from one of the bottles; a corresponding amount of nicotine, split into the two bottles, was proposed to dams under a yoked intake and an evidently unescapable way of exposure. Due to these presenting ways, "freely choosing" mothers consumed nearly double concentrated nicotine, from half volume of a bitter solution, than "yoked" dams; one could therefore expect nicotine and/or cotinine levels in the pups to differ accordingly, which was not the case.

The similar levels of nicotine in either group may be explained by a series of factors. First, we cannot exclude different adaptations that may have occurred in the epathic function of dams from either group, depending from a different pace of drug ingestion; second, and more likely, the drinking habits of dams may have

changed differentially due to free versus unavoidable offer of a psychoactive but bitter fluid. As just a possibility, “yoked-intake” mothers shall drink nicotine solutions as the only source of fluid when they are thirsty, while “freely choosing” dams may titrate nicotine intake, and dilute its psychoactive effects and perhaps its taste with water. It was far beyond our present purposes to provide such a detailed account, which could well be studied in further experiments. In our hands, the focus of present work was just to confirm the transfer of nicotine to pups as well as to calibrate the method for its quantification.

## CONCLUSIONS

We developed and validated a reliable method for quantification of nicotine and its metabolite cotinine in plasma samples by LC-MS/MS showing good results for linearity, precision, and recovery. Inter-day repeatability percentages (inter-day CV%) were below 12.0% for both nicotine and cotinine at LOQ values and below 9.0% at other concentration levels within the matrix calibration interval. Inter-day recovery ranged from 86.2% to 118.8% for both the analytes.

Finally, the present method was successfully employed to investigate the passage of nicotine from Wistar rat dams to their offspring through breast milk, to quantify its major metabolite, cotinine, and to ascertain whether the way of nicotine assumption (free-choice versus forced) may influence the nicotine results. In particular, we quantified their concentrations in plasma from pups whose mothers were orally administered with nicotine (two different nicotine consumption ways, “free-choice” versus “forced and yoked”) during lactation. We detected both analytes in the offspring plasma: nicotine was found in the 67% of plasma samples from free-choice drinking group ( $1.30 \pm 0.31$  ng/mL) and in the 60% of plasma samples from “forced / yoked-intake” group ( $1.19 \pm 0.62$  ng/mL); cotinine was found in all samples from free-choice drinking ( $1.92 \pm$

$0.77$  ng/mL) versus “forced/yoked-intake” ( $1.43 \pm 0.30$  ng/mL) groups. As expected, comparison between the two nicotine exposure groups showed no significant differences in nicotine and cotinine concentrations.

This is the first report, to our knowledge, that nicotine can be directly assessed in pups’ plasma, allowing to verify transfer with breast feeding. Available literature only dealt with physiological parameters of rodent pups whose mothers were directly injected with nicotine during lactation [32, 33]: as such, they assumed implicitly that nicotine passed to pups, or based a proof for such assumption with detection of cotinine alone [34]. Therefore, observed damage may well be ascribed to cotinine, not nicotine transfer. Our presently obtained data may have implication for future studies about perinatal exposure to nicotine, in that they do give an evidence-based support for evaluating the correlation between the way of exposure, the plasmatic concentration and the consequent effects and sequelae over the offspring.

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## Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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