Quantitative analysis of NNN in plasma by LC-MS/MS: a suitable alternative to urinary determination to assess the exposure to this important toxicant

N. Pluym[1], F. Sibul[1], X. Jin[2], J. Edmiston[2], M. Sarkar[2], G. Scherer[1], M. Scherer[1]

[1] Analytisch-Biologisches Forschungslabor GmbH, Semmelweisstr. 5, 82152 Planegg, Germany
[2] Altria Client Services, Center for Research & Technology, 601 E. Jackson St., Richmond, VA 23219, USA

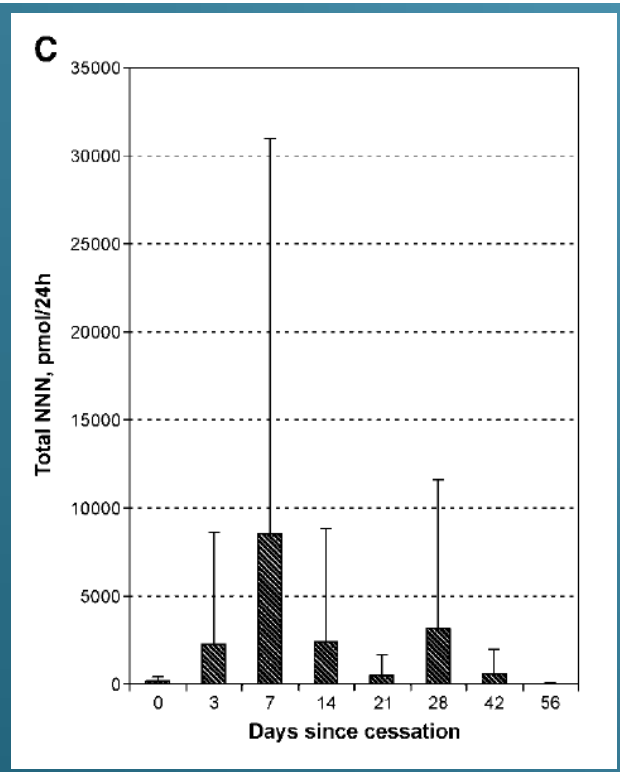
N-Nitrosonornicotine (NNN)

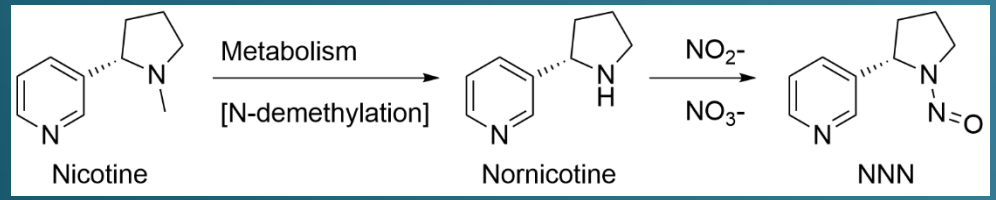
- NNN: IARC Group 1 carcinogen present in cigarette smoke and smokeless tobacco
- Exposure assessment by analysis of NNN in urine
- Several studies identified implausibly high NNN concentrations in urine, e.g. after quitting[1] or in nicotine patch users[2]
- NNN prone for artefactual formation by nitrosation of nornicotine with nitrite especially under acidic conditions[3]


Presence of the Carcinogen *N*'-Nitrosonornicotine in the Urine of Some Users of Oral Nicotine Replacement Therapy Products

Irina Stepanov, Steven G. Carmella, Anna Briggs, Louise Hertsgaard, Bruce Lindgren, Dorothy Hatsukami, and Stephen S. Hecht

Masonic Cancer Center and Transdisciplinary Tobacco Use Research Center, University of Minnesota, Minneapolis, Minnesota





[1]Stepanov, et al. (2009) Presence of the carcinogen N'-nitrosonornicotine in the urine of some users of oral nicotine replacement therapy products. Cancer Res.
[2]Stepanov, et al. (2009) Evidence for endogenous formation of N'-nitrosonornicotine in some long-term nicotine patch users. Nicotine Tob. Res.
[3]Mirvish, et al. (1977) Kinetics of Nornicotine and Anabasine Nitrosation in Relation to N'-Nitrosonornicotine Occurrence in Tobacco and to Tobacco-Induced Cancer. JNCI

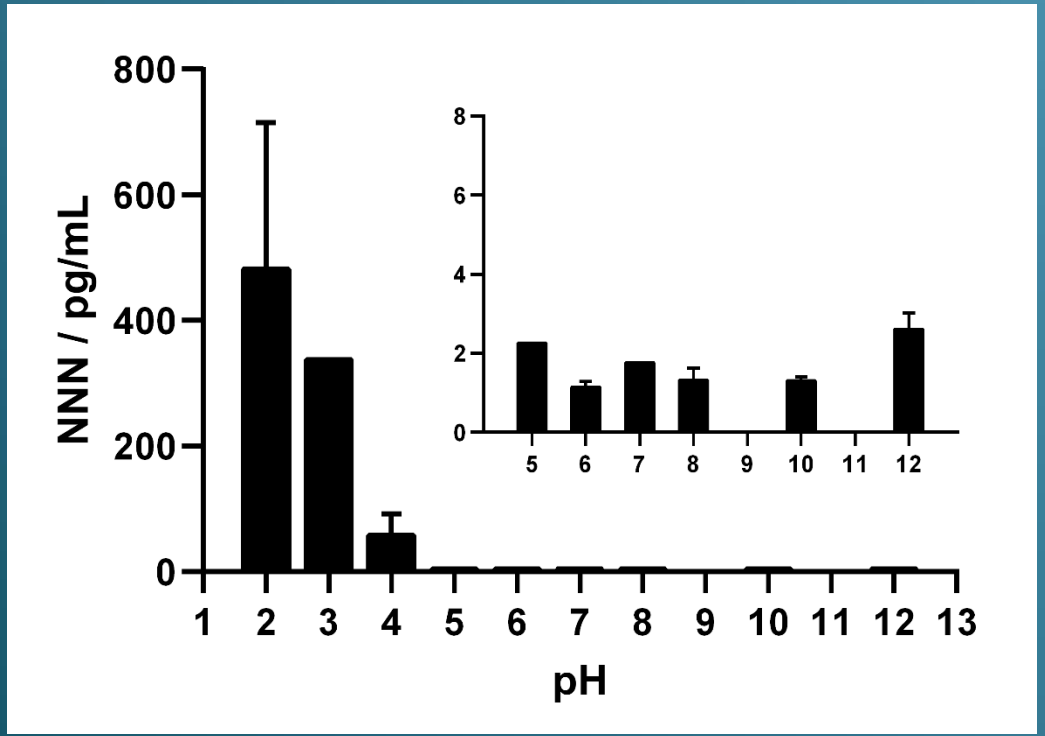
N-Nitrosonornicotine (NNN): Artefactual formation in urine

- Incubation experiments demonstrated rapid nitrosation at pH of 4 and below in urine
- High NNN yields observed over time for pH ≤ 5
- Artefactual NNN formation less likely at pH ≥ 6
- Urine pH as low as 5 may occur with various disease states (urinary tract/bladder infections; chronic kidney disease)[1]
- Measurement of urinary NNN may be confounded by artefactual formation (endogenously or exogenously during storage)

Urinary NNN may not necessarily reflect product-specific exposure

[1]Harlass, et al. (1990) The evaluation of urine pH in screening for asymptomatic bacteriuria in pregnancy. Military medicine

Incubation of nornicotine + NO_2^-/ NO_3^- at room temperature for 10 minutes

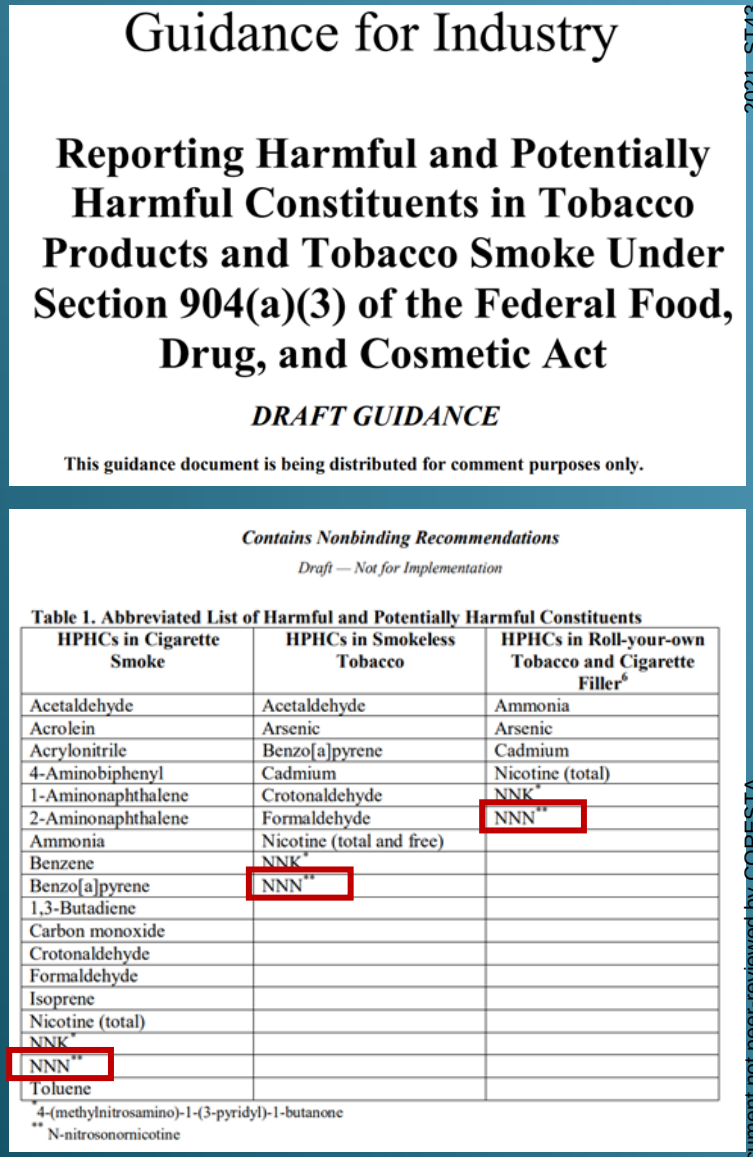


NNN in plasma
Analysis by LC-MS/MS

- NNN is part of the abbreviated list of harmful and potentially harmful constituents (HPHCs) in cigarette smoke, smokeless tobacco and roll-your-own tobacco
- Accurate and precise measurement of NNN exposure important for sound risk assessment
- Artefactual NNN formation less likely in blood due to consistent pH (7.40 ± 0.03)

Objective

Development of an analytical method with sufficient sensitivity for **plasma NNN determination** to assess **product-specific NNN exposure**



Guidance for Industry

Reporting Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke Under Section 904(a)(3) of the Federal Food, Drug, and Cosmetic Act

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only.

Contains Nonbinding Recommendations

Draft — Not for Implementation

Table 1. Abbreviated List of Harmful and Potentially Harmful Constituents

HPHCs in Cigarette Smoke	HPHCs in Smokeless Tobacco	HPHCs in Roll-your-own Tobacco and Cigarette Filler[6]
Acetaldehyde	Acetaldehyde	Ammonia
Acrolein	Arsenic	Arsenic
Acrylonitrile	Benzo[a]pyrene	Cadmium
4-Aminobiphenyl	Cadmium	Nicotine (total)
1-Aminonaphthalene	Crotonaldehyde	NNK*
2-Aminonaphthalene	Formaldehyde	NNN**
Ammonia	Nicotine (total and free)	
Benzene	NNK*	
Benzo[a]pyrene	NNN**	
1,3-Butadiene		
Carbon monoxide		
Crotonaldehyde		
Formaldehyde		
Isoprene		
Nicotine (total)		
NNK*		
NNN**		
Toluene		

* 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

** N-nitrosonornicotine

NNN in plasma
Analysis by LC-MS/MS

- Recently published LC-MS/MS method for rat serum NNN from the FDA served as starting point[1]

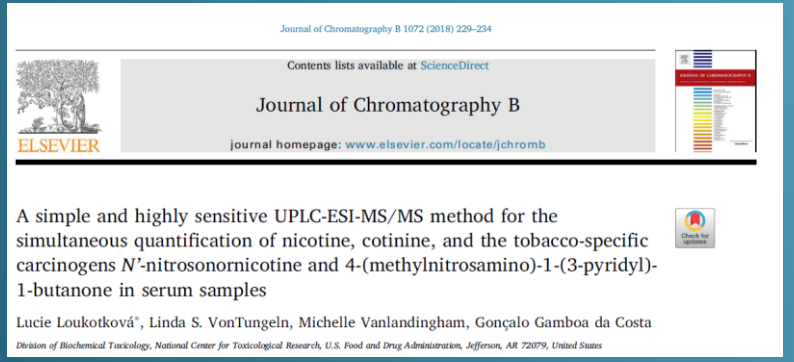
Plasma sample preparation

- Liquid-liquid extraction (LLE) of 1 mL plasma with methyl-tert-butylether (MTBE)
  - 1st LLE basic: 10 µL 10M NaOH + 100 µL brine + 2 mL MTBE
  - 2nd LLE acidic: reconst. of org. phase with 50 µL 4M formic acid + 150 µL MTBE
- Aq. phase evaporated to dryness and reconstituted in 50µL MeOH/H_2O (1:4)

LC-MS/MS analysis

- Shimadzu Nexera X2 UHPLC / Sciex 6500+ QTRAP MS/MS
- Injection of 10 µL on Acquity HSS T3 column (100x2.1mm; 1.8µm)
- Flow: 0.6 ml/min; Temp: 45 °C; run time: 5 min
  Eluents: A: 0.1%NH_4OAc in H_2O; B: 0.1% FA in ACN
- ESI^+; MRM: 178 → 148 *m/z* (Quantifier) / 182 → 152 (IS)

Full validation according to FDA guidelines[2]
LLOQ: 0.3 pg/mL (range: 0.3 – 1,000 pg/mL)


Journal of Chromatography B 1072 (2018) 229–234

Contents lists available at ScienceDirect

Journal of Chromatography B

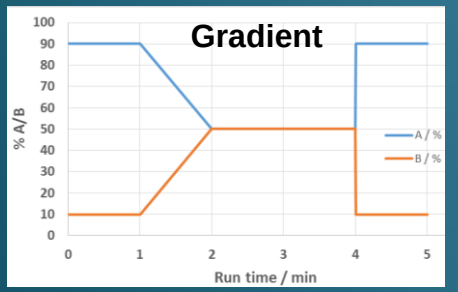
journal homepage: www.elsevier.com/locate/jchromb

ELSEVIER

A simple and highly sensitive UPLC-ESI-MS/MS method for the simultaneous quantification of nicotine, cotinine, and the tobacco-specific carcinogens *N'*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in serum samples

Lucie Loukotková*, Linda S. VonTungeln, Michelle Vanlandingham, Gonçalo Gamboa da Costa

Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States



[1]Loukotkova, L., et al. (2018) A simple and highly sensitive UPLC-ESI-MS/MS method for the simultaneous quantification of nicotine, cotinine, and the tobacco-specific carcinogens N'-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in serum samples. J Chrom B
[2] FDA. (2018) Bioanalytical Method Validation - Guidance for Industry

Application of the blood LC-MS/MS method for evaluation of NNN in smokeless tobacco users

Pharmacokinetic (PK) study – Study overview

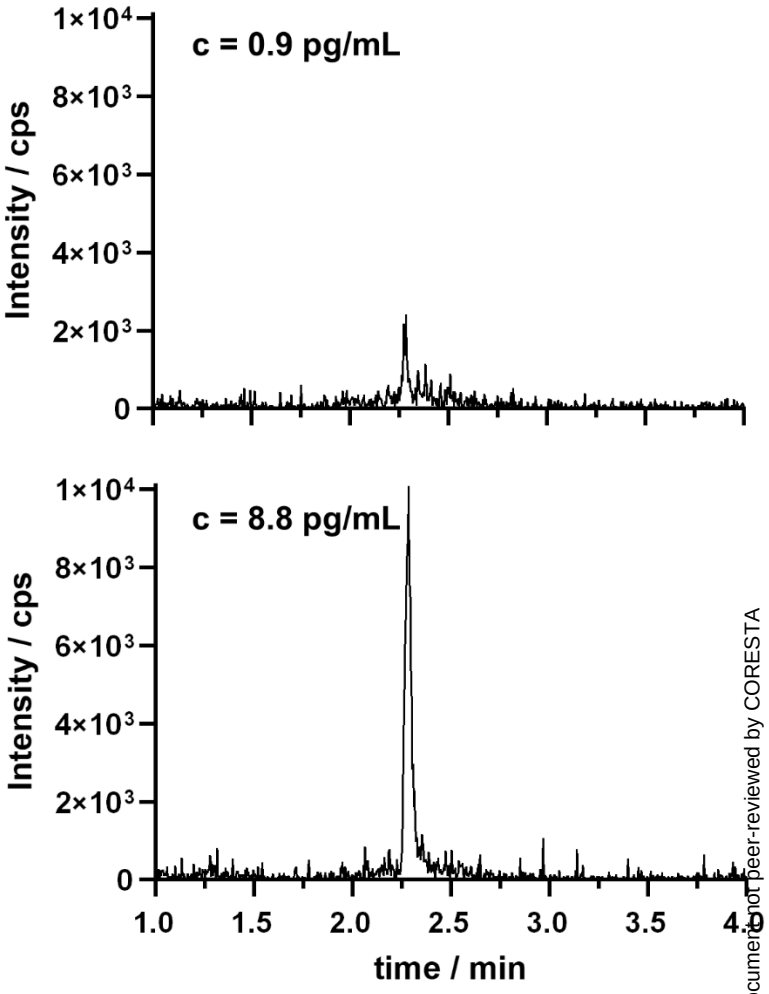
- Clinical study conducted in U.S. in accordance with Good Clinical Practice
- Study Product
  - Commercially available moist snuff tobacco (MST)
  - 12.41 mg/g nicotine
  - 1.77 µg/g NNN
  - pH: 7.7
- Study participants (N = 10):
  - Exclusive males
  - 28 – 59 years of age; BMI: 18.6 – 36.2 kg/m^2
- Overnight abstinence from tobacco use and then use of a defined portion of 2 g of the MST product for 40 min
- Sample collection: 15 blood draws during and post use
  - 0 (5 min pre-use), 5, 10, 15, 20, 25, 30, 35, 40, 45, 60, 90, 150, 240, 360 min
  - Total of 150 plasma samples for NNN and nicotine PK analysis

PK study – Results

PK parameter	NNN (mean ± SD; N = 10)	Nicotine (mean ± SD; N = 10)
AUC_{0-360}	917 ± 343 pg/mL*min	3095 ± 1507 ng/mL*min
$AUC_{0-\infty}$	1051 pg/mL*min	3698 ng/mL*min
C_{max}	7.1 ± 2.3 pg/mL	19.4 ± 9.1 ng/mL
t_{max}	32 ± 10 min	40 ± 14 min
$t_{1/2}$	98 min	122 min

- NNN > LLOQ (0.3 pg/mL) for 149 of 150 samples
- Difference in the pharmacokinetic parameters for Nic and NNN is proportionate to the amounts of Nic and NNN in the product
- Similar kinetics for both compounds (t_{max}, $t_{1/2}$)

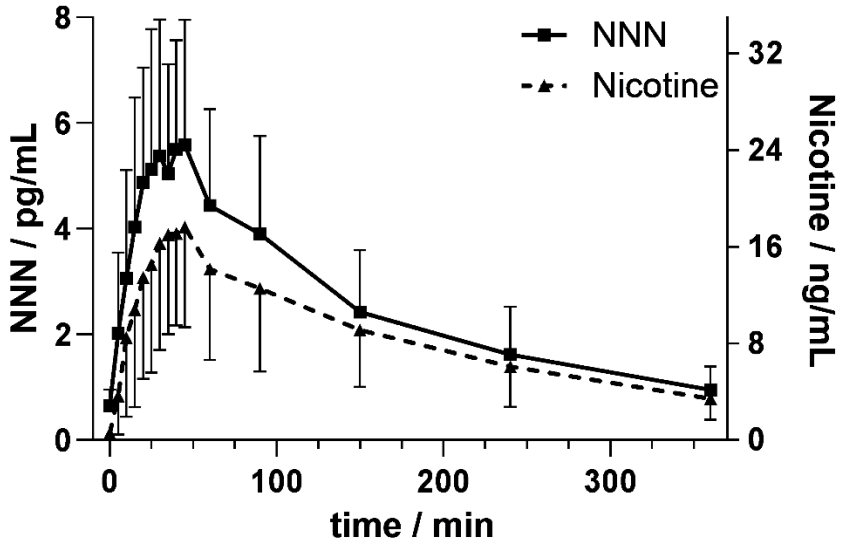
Chromatograms of NNN in plasma

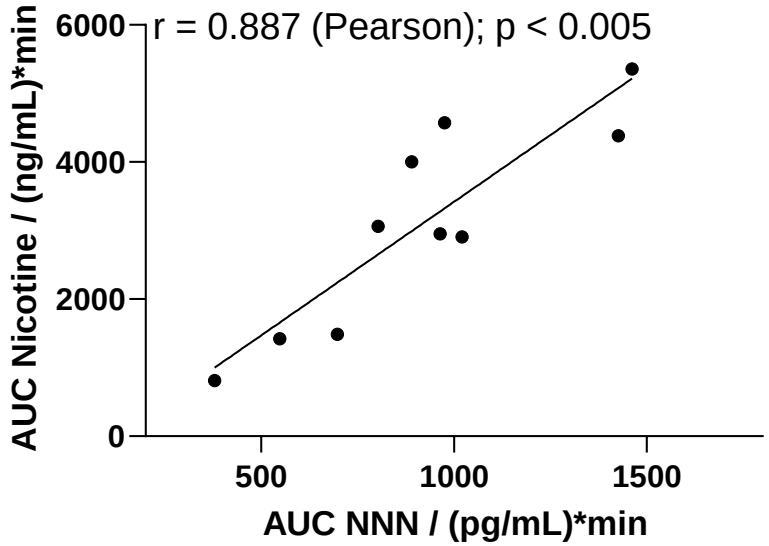


PK study – Results

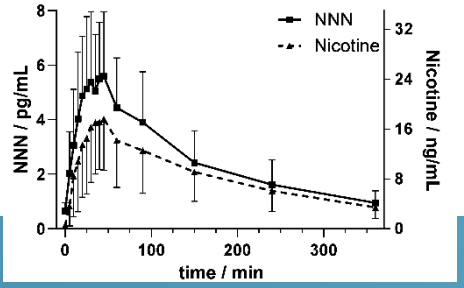
- Time courses of NNN resemble that of Nic
- Controlled use over 40 min resulted in concentration plateau between 20 and 40 min
- PK parameters of NNN and nicotine were highly correlated ($p < 0.005$):
  - $r = 0.803$ for C_{max}
  - $r = 0.887$ for AUC

High correlation between nicotine and NNN **plasma NNN** reflects **product-use specific uptake**





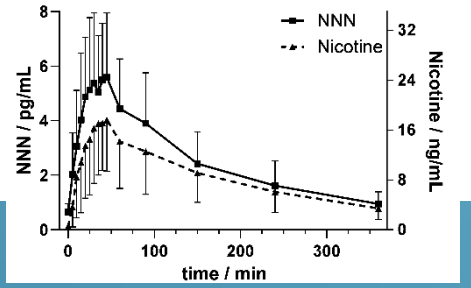
Summary and Conclusions



- High variability in urinary NNN most likely occurring due to nitrosation of nornicotine
- Need for robust determination of product-specific exposure to NNN
- We report:
  - A fit-for-purpose LC-MS/MS method to measure NNN in plasma
  - Correlation of NNN PK parameters to nicotine
- Overall these results demonstrate that this assay is a useful tool to assess product specific exposure to NNN

We conclude that plasma NNN is a fit-for-purpose biomarker to characterize product specific NNN exposure from tobacco products

Acknowledgement



- This study was funded by Altria Client Services

- **ABF**
  Dr. Filip Sibul, Marta Latawiec, Dr. Therese Burkhardt, Dr. Max Scherer, Prof. Gerhard Scherer

- **ALCS**
  Cathy Jin, Dr. Jeff Edmiston, Dr. Mohamadi Sarkar, Dr. Jianmin Liu

Altria

Contact

- Dr. Nikola Pluym
- ABF GmbH | Semmelweisstr. 5 | D-82152 Planegg
- Phone: +49-89-4114796-12
- Mail: nikola.pluym@abf-lab.com
- Homepage: www.abf-lab.com