TABLE OF CONTENT

6.1.3.1 Justification of Selection of Biomarkers of Exposure 4
6.1.3.1.1 Overview and Background 4
6.1.3.1.2 Selection of Biomarkers of Exposure to HPHCs 5
6.1.3.1.3 Assessment Methodology 13
6.1.3.1.4 Selected Biomarkers of Exposure – Rationale 14
6.1.3.1.4.1 Gas 14
6.1.3.1.4.1.1 Blood Carboxyhemoglobin and Exhaled Carbon Monoxide 14
6.1.3.1.4.2 Aliphatic Dienes 15
6.1.3.1.4.2.1 MHBMA – 1,3-Butadiene 15
6.1.3.1.4.3 Carbonyls 16
6.1.3.1.4.3.1 3-HPMA - Acrolein 16
6.1.3.1.4.3.2 3-HMPMA - Crotonaldehyde 17
6.1.3.1.4.4 Acid Derivatives 18
6.1.3.1.4.4.1 CEMA - Acrylonitrile 18
6.1.3.1.4.5 Epoxides 19
6.1.3.1.4.5.1 HEMA – Ethylene Oxide 19
6.1.3.1.4.6 Monocyclic Aromatic Hydrocarbons 20
6.1.3.1.4.6.1 S-PMA – Benzene 20
6.1.3.1.4.6.2 S-BMA - Toluene 22
6.1.3.1.4.7 Aromatic Amines 23
6.1.3.1.4.7.1 1-NA 23
6.1.3.1.4.7.2 2-NA 24
6.1.3.1.4.7.3 4-ABP 25
6.1.3.1.4.7.4 o-Toluidine 26
6.1.3.1.4.8 Tobacco Specific Nitrosamines 27
6.1.3.1.4.8.1 Total NNAL and Total NNN – NNK and NNN 27
6.1.3.1.4.9 Polycyclic Aromatic Hydrocarbons 29
6.1.3.1.4.9.1 Total 3-OH-B[a]P - Benzo[a]pyrene 29
6.1.3.1.4.9.2 Total 1-OHP - Pyrene 30

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6.1.3.1.4.10 Alkaloids 31
6.1.3.1.4.10.1 Nicotine and its Metabolites 31
6.1.3.1.4.11 Not Related to a Specific Chemical Entity 33
6.1.3.1.4.11.1 Cytochrome 1A2 33
6.1.3.1.4.11.2 Cytochrome 2A6 35
6.1.3.1.4.11.3 Urine Mutagenicity 36
6.1.3.1.5 Biomarkers of Exposure in Smokers and Non-Smokers Not Assessed in PMI's Clinical Studies 37
6.1.3.1.5.1 Isoprene 38
6.1.3.1.5.2 Formaldehyde 38
6.1.3.1.5.3 Acetaldehyde 39
6.1.3.1.5.4 AAMA and GAMA - Acrylamide 41
6.1.3.1.5.5 Ammonia 42
6.1.3.1.5.6 DNA and Protein Adducts 43
6.1.3.1.6 Biomarkers of Exposure in Non-Clinical Studies 44
6.1.3.1.6.1 COHb 46
6.1.3.1.6.2 3-HPMA 46
6.1.3.1.6.3 S-PMA 47
6.1.3.1.6.4 NNAL 47
6.1.3.1.6.5 CEMA 47
6.1.3.1.6.6 Nicotine Metabolites 47
6.1.3.1.6.7 Menthol 48
6.1.3.1.6.8 Not Related to a Specific Chemical Entity 49
6.1.3.1.6.8.1 Cytochrome 1A2 49
6.1.3.1.6.8.2 Cytochrome 1A1 and 1B1 49
6.1.3.1.7 Published Data on BoExp Levels in Smoker, Non-Smokers, and Quitters 49
6.1.3.1.8 References 67

TABLE OF TABLES

Table 1 Lists of HPHCs Considered to Select Toxicants 5

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Table 2 List of HPHCs Considered for PMI's Clinical Assessment Program, with the Chemical Classes, Presence in Gas/Vapor (GVP) or Particulate Phases (PP) and FDA's Risk Categories.....9

Table 3 List of PMI's Selected HPHCs with their Corresponding Biomarkers of Exposure 10

Table 4 Reported Half-lives of HPHC / BoExp in the Literature 12

Table 5 Representative Levels of BoExp in Smokers and Non-Smokers – Literature Review of Cross Sectional Studies.....50

Table 6 Representative Reductions of Levels of BoExp Achieved upon Smoking Cessation – Literature Review of Longitudinal Studies 61

TABLE OF FIGURES

Figure 1 Ranges at which HPHCs are Formed or Transferred during Tobacco Combustion 7

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6.1.3.1 Justification of Selection of Biomarkers of Exposure

6.1.3.1.1 Overview and Background

Cigarette smoke is a complex mixture composed of 76% air, 17% gases such as CO, CO_2, H_2, CH_4, 2% H_2O and 5% particulate matter, with thousands of chemical species present in both the gas and the particulate phases of cigarette smoke (Senneca 2007). More than 6000 constituents have been identified as of today (Rodgman 2013). A certain number of these constituents, defined as tobacco toxicants or "harmful and potentially harmful constituents" (HPHCs) have been selected and summarized in several guidance documents by regulatory agencies or health organizations, aiming at prioritizing those that are of the highest concern, and should, therefore, be monitored, reported, and/or lowered (FDA (Food and Drug Administration) 2012, Health Canada 2013, Health Canada Modified 2011, U.S. Department of Health and Human Services 2012c, WHO Study Group 2015, WHO Study Group 2008) (Table 1). The existence of these different and differing lists of HPHCs reflects both the fact that a full analytical characterization of the aerosol/smoke matrix is not possible due to its chemical complexity and that the purposes and priorities of the issuing regulatory agencies or health organizations differ.

Consequently, in order to demonstrate that a candidate MRTP reduces exposure to HPHCs, the PMI assessment focused on measuring changes in the levels of a set of relevant HPHCs drawn from those lists. Evidence of reduced exposure to this set of HPHCs lays the foundation for demonstrating that THS use has the potential to reduce the risk of smoking related diseases compared with smoking cigarette.

The ability to measure exposure to HPHCs *in vivo* is, however, limited by various factors, such as 1) the specificity of the constituents to the source of exposure (i.e conventional cigarette smoke exposure) with minor or non-existent contribution of other sources of exposure (e.g., environmental exposure, diet) to avoid confounding factors in the results, and 2) the availability of easily detectable biomarkers using reliable, reproducible, and precise analytical methods.

In a non-clinical and a clinical setting, biomarkers of exposure (BoExp) to HPHCs or their metabolites in biological fluids, provide direct quantitative evidence of the presence of HPHCs, or their metabolites, in the body. The selection of which BoExp to HPHCs to be measured must be based on existing criteria and, more importantly, needs to take into account the nature and specification of the candidate MRTP assessed.

The criteria and justification for the selection of the HPHCs and their BoExp assessed as part of Tobacco Heating System (THS) assessment program are detailed below as well as in section 6.1.3.1.2.

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Table 1 Lists of HPHCs Considered to Select Toxicants

Abbreviated Name of the List	Description of the List	Reference
FDA-93	Established list of 93 HPHCs in tobacco products	(U.S. Department of Health and Human Services 2012c)
FDA-18	Abbreviated list of 18 HPHCs to be reported to FDA	(FDA (Food and Drug Administration) 2012)
PMI-58	List of 58 HPHCs measured at PMI	(Roemer 2012, Roemer 2004) [a]
HC	List of 44 constituents in the mainstream smoke of tobacco products to be reported to Health Canada [b]	(Health Canada 2013, Health Canada Modified 2011)
WHO-39	Non-exhaustive list of 39 priority toxic contents and emissions of tobacco products	(WHO Study Group 2015) [b]
WHO-18	Initial list of 18 priority toxicants	(WHO Study Group 2008)
WHO-9	Mandated lowering list of 9 toxicants	(WHO Study Group 2008)

[a] Partially listed; [b] Based on Hoffmann analytes list (Hoffmann 1986, Rodgman 2013). Abbreviations: Food and Drug Administration (FDA); Health Canada (HC); Philip Morris International (PMI); World Health Organization (WHO)

6.1.3.1.2 Selection of Biomarkers of Exposure to HPHCs

More than a hundred HPHCs of various chemical classes are recognized as being associated with the development of smoking-related diseases and most of these HPHCs are represented in the guidance of the U.S. Food and Drug Administration (FDA) on "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 (FDA-93 list) (U.S. Department of Health and Human Services 2012c). These HPHCs have been classified in the 5 major risk and toxicity categories defined by the FDA (i.e., carcinogen (CA), respiratory toxicant (RT), cardiovascular toxicant (CT), reproductive or developmental toxicant (RDT) or addictive (AD) (U.S. Department of Health and Human Services 2012c) (Table 2).

It is not possible to measure all of those potentially toxic constituents in humans, due to constraints in availability of validated, reliable methods, or simply the absence of suitable BoExp. However, the FDA and WHO have established shortened lists of HPHCs recommended to be measured and reported for tobacco products (FDA-18) (FDA (Food and Drug Administration) 2012), or recommended for mandated lowering in cigarette smoke (WHO-9) (WHO Study Group 2008) (Table 1), which served as the main references for the selection of HPHCs assessed in PMI non-clinical and clinical studies.

Most HPHCs are formed by pyrosynthesis during cigarette combustion (Adam 2009, Baker 1987, Borgerding 2005, Torikai 2004, White 2001). The chemical composition of smoke after combustion thus depends on 3 factors (Senneca 2007):

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- the chemical composition of the tobacco used, which is determined by the organic composition of the tobacco leaves that is formed during plant growth
- post-harvest handling processes of curing, aging and fermenting, and the manufacturing of cigarettes, including the blending, and
- the reaction conditions occurring during the combustion of the tobacco during smoking.

While adaptations of the post-harvest handling processes of curing, aging and fermenting, as well as manufacturing and blending of the cigarettes may reduce levels of selected HPHCs, numerous studies have now shown that heating tobacco to temperatures below pyrolysis and combustion temperatures has the potential to reduce or eliminate toxicants contained in cigarette smoke on a broader scale (deBethizy 1990, Forster 2015, Schorp 2012, Smith 1996, Urban 2012, Werley 2008). Heating instead of burning tobacco, typically at temperatures lower than 350°C, is sufficient to release nicotine, but not high enough to cause pyrolysis (Forster 2015, Piade 2013), resulting in a substantially reduced formation of HPHCs and therefore a less complex aerosol composition than that found in cigarette smoke (Schorp 2012, Zenzen 2012). PMI's THS heats the tobacco to temperatures < 350°C and therefore, unlike CC, avoids combustion, thereby reducing or eliminating the formation of HPHCs and their transfer into the aerosol (Baker 2006a). For a heat-not-burn product, the HPHCs considered for assessment take into account their toxicity, the diverse temperature ranges for their formation, and include markers of evaporation and distillation such as NNN, NNK, 4-ABP, 1- and 2-NA as well as markers of combustion such as CO and pyrene. Figure 1 provides an overview on temperature ranges at which selected HPHCs are formed or transferred into cigarette smoke.

To date, the only known tobacco specific HPHCs, besides nicotine, are 4 (Methylnitrosamino)-1-(3-pyridyl)-1butanone (NNK) and N-nitrosonornicotine (NNN). Other HPHCs can arise from other sources. For example, acrolein occurs naturally in foods and is formed during the combustion of fossil fuels (including engine exhausts), wood and tobacco and during the heating of cooking oils (IARC (International Agency for Research on Cancer) 1995). The PMI clinical exposure assessment program was designed to assess the exposure to HPHCs in a controlled, confined setting to avoid exposure from sources other than tobacco smoke to the extent possible and evaluate how exposure levels change when assessed in a more near real-world or ambulatory setting [Section 6.1.3.2 – Summary of BoExp Assessment; and Section 6.2.2.1 – Summary of PK/PD Assessment, Table 4.1, Clinical studies of THS 2.2: study overview]. Importantly, each BoExp chosen should be sensitive to smoking status (dose-response), with its level increasing according to smoking intensity and decreasing after smoking cessation. The section 6.1.3.1.7 provides a tabulated summary of representative differences of levels of BoExp to HPHCs observed in smokers and non-smokers, as well as reductions in exposure measured when smokers stop smoking as reported in the literature.

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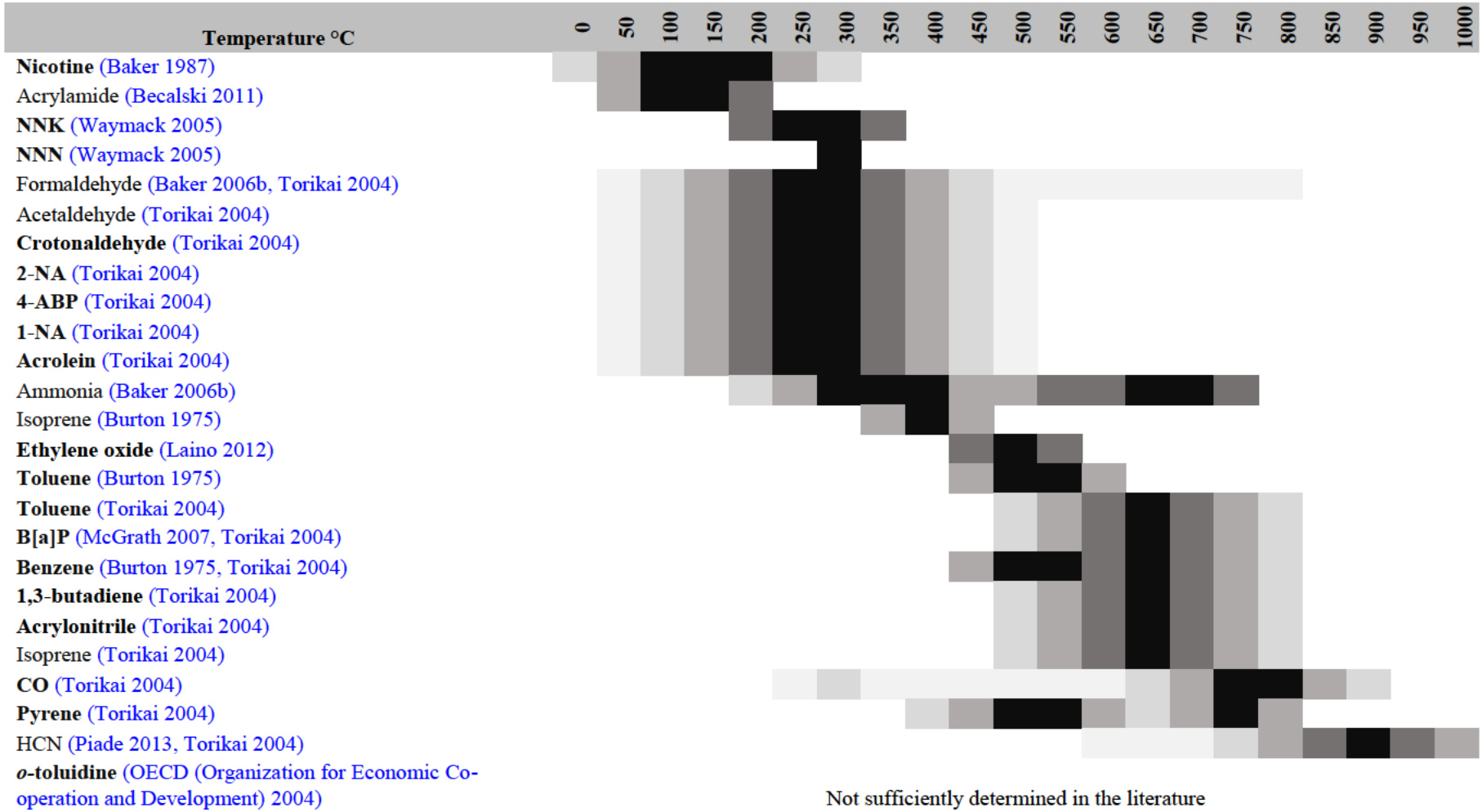


Figure 1 Ranges at which HPHCs are Formed or Transferred during Tobacco Combustion

Abbreviations: 4-aminobiphenyl (4-ABP); 1-aminonaphthalene (1-NA); 2-aminonaphthalene (2-NA); benzo[a]pyrene (B[a]P); hydrogen cyanide (HCN) harmful and potentially harmful constituents (HPHC); 4-(Methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK); N-Nitrosonornicotine (NNN). HPHCs assessed in PMI's studies are highlighted in **bold**.

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To allow assessment of exposure reduction in a reasonably short period of time, HPHCs and their BoExp must also exhibit an apparent elimination half-life short enough to be suitable for assessment in confined clinical studies, while covering a range from hours to multiple days to help differentiating exposure between smoking CC and using MRTP (or other sources) in longer-term ambulatory studies.

In summary, the criteria applied by PMI that qualify HPHCs and their related BoExp to be assessed are:

1. The HPHC selected are representative of a variety of chemical classes and organ toxicity classes as defined by the FDA (carcinogen, cardiovascular toxicant, respiratory toxicant, reproductive and development toxicant, addiction potential)
2. The HPHC reflects a specific toxic exposure or is a reliable surrogate of exposure to HPHCs
3. The HPHC assessed cover a broad range of formation temperatures
4. The HPHC is specific to smoking with other sources being minor or non-existent
5. The BoExp to a HPHC is reliably detectable using validated, reproducible, precise analytical methods
6. The BoExp to a HPHC has a half-life that is suitable with the schedule of assessments

Exposure to 17 HPHCs (including nicotine) were evaluated in PMI clinical studies, by either measuring the parent compound (e.g., 4-aminobiphenyl), by measuring one or several of their metabolites, or by using a surrogate BoExp, representative of a chemical class of compounds. For example, 1-hydroxypyrene, a BoExp for pyrene that is not on the FDA or WHO lists of HPHCs, can be used as a surrogate marker for polycyclic aromatic hydrocarbons (PAH).

Table 2 provides the final list of HPHCs measured in PMI clinical studies and their allocation to chemical classes, presence in GVP or TPM, as well as their risk categorization by the FDA, while Table 3 presents those HPHCs with their selected BoExp, as well as whether they are or not part of the FDA-18 and WHO-9 lists of HPHCs.

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Table 2 List of HPHCs Considered for PMI's Clinical Assessment Program, with the Chemical Classes, Presence in Gas/Vapor (GVP) or Particulate Phases (PP) and FDA's Risk Categories

PMI' List of HPHCs Covered in Clinical Studies	BoExp Coverage of FDA Toxicity Classes					
	None	**CA**	**CT**	**RT**	**RDT**	AD
Gas:						
carbon monoxide (GVP)	-	-	-	-	●	-
Aliphatic dienes:						
1,3-butadiene (GVP/)	-	●	●	-	●	-
Carbonyls:						
acrolein (GVP)	-	-	●	●	-	-
crotonaldehyde (GVP)	-	●	-	-	-	-
Acid derivatives:						
acrylonitrile (GVP)	-	●	-	●	-	-
Epoxides:						
ethylene oxide (GVP)	-	●	-	●	●	-
Inorganic compounds:						
none	-	-	-	-	-	-
Monocyclic aromatic hydrocarbons:						
benzene (GVP)	-	●	●	-	●	-
toluene (GVP/PP)	-	-	-	●	●	-
Aromatic amines:						
1-NA (PP)	-	●	-	-	-	-
2-NA (PP)	-	●	-	-	-	-
4-ABP (PP)	-	●	-	-	-	-
o-toluidine (PP)	-	●	-	-	-	-
N-nitrosamines:						
NNK (PP)	-	●	-	-	-	-
NNN (PP)	-	●	-	-	-	-
Phenols:						
none	-	-	-	-	-	-
Polycyclic aromatic hydrocarbons (PAH):						
B[a]P (PP)	-	●	-	-	-	-
pyrene (PP) #	●	-	-	-	-	-
Metals and Elements:						
none	-	-	-	-	-	-
Alkaloids:						
nicotine (TPM)	-	-	-	-	●	●

Abbreviations: Food and Drug Administration (FDA); 4-aminobiphenyl (4-ABP); 1-aminonaphthalene (1-NA); 2-aminonaphthalene (2-NA); benzo[a]pyrene (B[a]P); harmful and potentially harmful constituents (HPHC); gas/vapor phase (GVP); 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); N-Nitrosonornicotine (NNN); Philip Morris International (PMI); Particulate phase (PP); Total particulate matter (TPM).

Pyrene is not listed as a HPHC by FDA, but is nevertheless listed here because its metabolite 1-OHP serves as a surrogate for PAH in general.

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Table 3 List of PMI's Selected HPHCs with their Corresponding Biomarkers of Exposure

HPHC	WHO-9	FDA-18	PMI's Selection of BoExp for Clinical Studies
Acrolein	●	●	3-hydroxypropylmercapturic acid (3-HPMA)
Acrylonitrile		●	2-cyanoethylmercapturic acid (CEMA)
4-Aminobiphenyl		●	4-aminobiphenyl (4-ABP)
Benzene	●	●	S-phenylmercapturic acid (S-PMA)
Benzo[a]pyrene	●	●	3-hydroxybenzo[a]pyrene (3-OH-B[a]P)
1,3-Butadiene	●	●	monohydroxybutenylmercapturic acid (MHBMA)
Carbon monoxide (CO)	●	●	blood carboxyhemoglobin (COHb) * and exhaled CO * (COex) *
Crotonaldehyde		●	3-hydroxy-1-methylpropyl-mercapturic acid (3-HMPMA)
Ethylene oxide			2-hydroxyethylmercapturic acid (HEMA)
1-Aminonaphthalene		●	1-aminonaphthalene (1-NA)
2-Aminonaphthalene		●	2-aminonaphthalene (2-NA)
Nicotine		●	nicotine equivalents (NEQ) Plasma Nicotine and continine*
NNK	●	●	total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (total NNAL)
NNN	●	●	total N-nitrosonornicotine (total NNN)
o-Toluidine			*o*-toluidine
Pyrene #			total 1-hydroxypyrene (1-OHP)
Toluene		●	S-benzylmercapturic acid (S-BMA)

* Those BoExp are the only ones not measured in urine

Pyrene is not listed as a HPHC by FDA, but is nevertheless listed here because its metabolite 1-OHP serves as a surrogate for PAH in general.

Abbreviations: Food and Drug Administration (FDA); harmful and potentially harmful constituents (HPHC); World Health Organization (WHO).

Out of the 17 BoExp in PMI's list, 14, including nicotine, are listed in the FDA-18 list (FDA (Food and Drug Administration) 2012) and 7 are listed on the WHO-9 list (WHO Study Group 2008) (Table 3). Four of the HPHCs listed by the FDA (i.e., isoprene, formaldehyde, acetaldehyde and ammonia), were not included in the set of HPHCs assessed in PMI clinical studies due to a lack of reliable methods or meaningful BoExp to assess the difference in exposure between smokers and non-smokers, or because these constituents are present due to other sources and therefore are subject to strong confounding factors (see section 6.1.3.1.5).

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Furthermore, 2 HPHCs, which are in the FDA-93 list (U.S. Department of Health and Human Services 2012c) (i.e., ethylene oxide, and *o*-toluidine,) have also been included in current PMI's clinical assessment studies, because bioanalytical methods were available to measure them reliably (or one of their metabolite). Finally, pyrene, which is not in any of the HPHC lists, has also been selected, since its metabolite 1-OHP is an accepted and widely used marker to monitor exposure to PAHs, several of which are known to be carcinogenic.

The terminal half-lives of the BoExp to HPHCs measured range from short (up to 6 hours), medium (up to 1 day) to long (up to 2 weeks or more), as reported in the literature (Table 4).

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Table 4 Reported Half-lives of HPHC / BoExp in the Literature

Smoke Constituent [smoke phase]	Biomarker [Matrix]	Biomarker $t_{1/2}$ [Ref]
1,3-Butadiene [gas]	Monohydroxybutenyl-mercapturic acid (MHBMA)	8.6h (Sarkar 2005) 5-9h (Gregg 2013)
1-Aminonaphthalene [particulate]	1-Aminonaphthalene (1-NA)	Not reported
2-Aminonaphthalene [particulate]	2-Aminonaphthalene (2-NA)	Not reported
4-Aminobiphenyl [particulate]	4-Aminobiphenyl (4-ABP)	31h (in rats) (Karreth 1991)
Acrolein [gas]	3-Hydroxypropylmercapturic acid (3-HMPMA)	5-9h (Gregg 2013)
Acrylonitrile [gas]	2-Cyanoethylmercapturic acid (CEMA)	7-9h (Jakubowski 1987)
Benzene [gas]	S-Phenylmercapturic acid (S-PMA)	9h (Benowitz 2009, Boogaard 1996, Gregg 2013, van Sittert 1993) $t_{1/2}$ α-phase: 9h (van Sittert 1993) $t_{1/2}$ β-phase: 45h (van Sittert 1993)
Benzo[a]pyrene [particulate]	3-Hydroxybenzopyrene (3-OH-B[a]P) [Urine]	2.5-4.3h (Chien 2012)
Crotonaldehyde [gas]	3-Hydroxy-1-methylpropyl-mercapturic acid (3-HMPMA or HBMA)	5-9h (Gregg 2013)
Ethylene Oxide [gas]	2-Hydroxyethyl mercapturic acid (HEMA)	< 5h (Haufroid 2007)
NNK [particulate]	Total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (Total NNAL)	$t_{1/2}$ α-phase: 14.3h* (Goniewicz 2009) $t_{1/2}$ β-phase: 10.3 days (Goniewicz 2009) Up to 45.2 days (Hecht 1999b)
NNN [particulate]	Total N-nitrosonornicotine (NNN)	< 24h (Stepanov 2009a) 27 min (patas monkey) (Upadhyaya 2002)
Pyrene [particulate]	Total 1-hydroxypyrene (1-OHP)	12h (Viau 1995) < 10h (St Helen 2012)
o-Toluidine [gas]	*o*-Toluidine	4h (plasma) (Herdevall 2003)
Toluene [gas]	S-benzylmercapturic acid (S-BMA)	< 10h (Inoue 2004)

* Half-life evaluated by non-compartmental analysis, in clinical settings was estimated to be 9.1 days.
Abbreviations: 4-(Methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK); N-Nitrosonornicotine (NNN); bibliographic reference (Ref).

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All BoExp were analyzed from 24-hour urine collections, both during confinement and ambulatory settings, except for blood COHb and exhaled breath CO, using validated methods (U.S. Department of Health and Human Services 2012c). Details on the bioanalytical methods used can be found in [Section 6.1.3.3 Bioanalytical Methods].

Not all smoke constituents can be assessed in clinical studies, thus PMI has selected BoExp on a "fit-for-purpose" approach. The data obtained with these BoExp across multiple clinical studies and with different populations, both in Confinement and Ambulatory settings, together with the product use behavior when switching from CC to THS, provide a comprehensive scientific foundation to conclude that users of THS are exposed to lower levels of HPHCs as compared with the levels of those HPHCs in CC smoke. The non-clinical data on exposure further strengthen the clinical conclusions.

6.1.3.1.3 Assessment Methodology

In urine, the measurement of BoExp to HPHCs is an effective, feasible and reliable way to estimate a smoker's exposure to tobacco smoke constituents and provides direct, quantitative evidence of the presence of HPHCs, or their metabolites, in the body with limited burden for the study subject and, this, without having to take into account smoking behavior or counting the number of tobacco articles smoked (Gregg 2006, Hatsukami 2003, Scherer 1999). Such an approach has been established for many years to assess the efficacy of control measures in occupational settings and is now a commonly used tool (Biomonitoring) to determine chemical exposure in the general population (Arnold 2013).

For most HPHCs, little, if any, intact parent compounds can be determined in blood. This can be due to their rapid metabolic transformation and/or adduct formation with a variety of blood/cellular components, such as nucleotides, proteins and glutathione. This renders their measurement, including that of all of their metabolites, impossible. Also, the limited information on the half-lives in blood for the majority of BoExp assessed to demonstrate reduced exposure to HPHCs selected in PMI studies further limits the use of blood measurements. Furthermore, clinical blood samples remain biologically active unless care is given to inhibit enzyme activity through sample modification and deep freezing, which may be time-, pH- and temperature-sensitive and only partially effective. Therefore, from an analytical perspective, the use of BoExp in 24hrs urine overcomes many limitations compared to measurement of HPHCs in blood.

For the BoExp assessed in PMI clinical studies (Table 3) a sufficient volume of urine or blood sampling was requested to perform an initial assay, sample reproducibility testing, and any re-assays that were analytically deemed necessary. In case of urinary samples for BoExp measurement, no modification of the urine was required to ensure sample stability. The stability of assays to measure BoExp was suitable for the duration of the clinical studies, and the analytical method ranges were deemed adequate to cover the needs of the studies. Bioanalytical methods and their respective validation parameters used in the THS clinical assessment program are described in Section 6.1.3.3.

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6.1.3.1.4 Selected Biomarkers of Exposure – Rationale

This section describes each BoExp that has been selected for and assessed in PMI clinical studies. For more detailed information on each HPHC and related BoExp, a factsheet with their main characteristics is available in Appendices A6.1.3.1 to A6.1.3.25.

6.1.3.1.4.1 Gas

6.1.3.1.4.1.1 Blood Carboxyhemoglobin and Exhaled Carbon Monoxide

Carbon monoxide (CO) has been classified as a reproductive or developmental toxicant by the FDA (U.S. Department of Health and Human Services 2012c), and is part of its list of 18 HPHCs to report (FDA (Food and Drug Administration) 2012, U.S. Department of Health and Human Services 2012c). It is also classified as a cardiovascular and respiratory toxicant by the Agency for Toxic Substances and Disease Registry and the U.S. Environmental Protection Agency (U.S. Department of Health and Human Services 2012d, U.S. Environmental Protection Agency (EPA) 2010). It is one of the 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering, based on its likely involvement in the progression of atherosclerosis and other cardiovascular diseases (WHO Study Group 2008). The Agency for Toxic Substances and Disease Registry report that carboxyhemoglobin (COHb) levels ≥2.4% may have adverse cardiovascular effects in subjects with compromised cardiovascular function (U.S. Department of Health and Human Services 2012d). Similarly, the WHO Environmental Health Criteria 213 on CO states that a carboxyhemoglobin level of 2.5% should not be exceeded to prevent hypoxic effects on the non-smoking population with coronary artery diseases (United Nations Environment Programme (UNEP) 1999), which was further emphasized in a guideline by the WHO for indoor air quality (WHO (World Health Organization) 2010). Both COHb and COex are listed in the report of the Institute of Medicine on the "Scientific standards for studies on modified risk tobacco products" as being "representative exposure biomarkers related to tobacco carcinogens and toxicants" (IOM (Institute of Medicine) 2012). The toxic effects of CO are the result of its binding to heme proteins, thereby affecting their function, including transport of oxygen (U.S. Environmental Protection Agency (EPA) 2010).

Besides exposure by endogenous production, the general population is exposed to varying levels of CO through inhalation of air, since CO is a temporary atmospheric pollutant in urban areas, mainly from the exhaust of internal combustion engines, but also from incomplete combustion of various other fuels.

Because of the very strong affinity of CO to hemoglobin, its release is slow, with most of the CO eventually released through the lungs, which can then be measured as exhaled CO (COex) (Wu 2005). COHb half-life is estimated to range from 1 to 4 hours, depending on the ventilation rate and the physical activity (Scherer 2006a).

It has been shown that the alveolar CO pressure is proportional to the concentration of COHb in blood, therefore, COex, in principle, accurately reflects blood COHb (Deveci 2004, Fife 2001, Scherer 2006a). The advantage of measuring COex is that it is not invasive, however, it may be unreliable depending on the sampling time, as it has been shown to not have a linear correlation with COHb shortly after a cigarette consumption

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(Scherer 2006a). The estimated COex half-life is of 4.5h (Sandberg 2011). COHb and CO in breath can be confounded by many other factors such as diet, physical exercise, inflammatory diseases and time of the day (Sandberg 2011).

Because of the endogenous production of and environmental exposure to CO, as well as the influence of physical activity, background levels of COHb and COex in non-smokers are variable. CO exposure from environmental sources can result in expired CO levels of 2–6 ppm, depending on the extent of exposure to traffic exhaust and other pollution (Society for Research on Nicotine and Tobacco Subcommittee on Biochemical Verification 2002). Thus, cut-off values have been established to distinguish smokers from non-smokers. This cut-off is 8-10 ppm, irrespective of the physical activity, meaning that any result above 10 ppm is considered as a consequence of cigarette smoking (Society for Research on Nicotine and Tobacco Subcommittee on Biochemical Verification 2002). Scherer has suggested that to differentiate smokers from non-smokers with acceptable sensitivity and specificity, cut-off levels should be 1.6-2.0% COHb (Scherer 2006a).

The relation between cigarette consumption and COHb or COex levels has been investigated in many studies (reviewed in (Scherer 2006a)). The coefficients of correlation are in the range of 0.3–0.8. Thus, the number of cigarettes smoked explains about 10–60% of the variability in the COHb or COex concentrations (Scherer 2006a). The coefficients of correlation are usually slightly higher when the number of cigarettes smoked prior to sampling was specifically assessed rather than using the average daily cigarette consumption figures (Scherer 2006a). A recent study in which smokers consuming 20 cigarettes per day (cpd) or more were forced to smoke exactly 20, 15, 5 or 0 cpd during a 5-day confinement period showed a dose-relationship between the number of cigarettes and the levels of COex or COHb (i.e., 22.9, 16.7, 12.6 and 2.0 ppm, respectively, for COex and 4.3, 3.3, 2.7 and 1.0 %, for COHb) (Theophilus 2015).

COHb levels are correlated with the number of cigarettes smoked per day, and to a lesser extent with the tar yield of cigarettes (Lindner 2011). For an average smoker, 24 hours should be sufficient to reach the cut-off level for COex (Society for Research on Nicotine and Tobacco Subcommittee on Biochemical Verification 2002).

Further details on the metabolism of CO are discussed in in the carbon monoxide factsheet [Appendix A6.1.3.1; Factsheet Carbon Monoxide].

6.1.3.1.4.2 Aliphatic Dienes

6.1.3.1.4.2.1 MHBMA – 1,3-Butadiene

IARC lists 1,3-butadiene in the Group 1 agents, i.e., carcinogenic to humans (IARC (International Agency for Research on Cancer) 2008a). It has been classified as a carcinogen, cardiovascular toxicant and reproductive or developmental toxicant by the FDA (U.S. Department of Health and Human Services 2012c) and is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012). 1,3-butadiene is also one of the 9 HPHCs recommended by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke, based on its high ranking with respect to cancer hazards (WHO Study Group 2008) .

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The main source of exposure to 1,3-butadiene for the general population, except for tobacco smoke, is the incomplete combustion of petroleum-derived fuels (European Chemical Agency (ECHA) 2002). Indoors, potential sources include release from materials composed of rubber, plastics and resins, such as carpet backings or food packaging. The half-life of MHBMA is estimated to be of around 9 hours (Sarkar 2005).

Smoke exposure was shown to increase several biomarkers of 1,3-butadiene exposure in both rats and humans (Urban 2003). In particular, an increased excretion of 1,2-di-hydroxybutyl mercapturic acid (DHBMA) and MHBMA was observed in smokers vs. non-smokers. Due to the lower background value for MHBMA, the dynamic effect of smoking was more pronounced for MHBMA (7-fold increase) than for DHBMA (40% increase). Reported urinary levels of MHBMA in smokers and non-smokers differ by 2- to almost 40-fold (Bergen 2015, Ding 2009, Eckert 2011, Jain 2015, Lindner 2011, Sarkar 2005, Scherer 2006b, Schettgen 2009, Shepperd 2013), but are generally around 10-fold higher in smokers. A recent study in which smokers consuming 20 cpd or more were forced to smoke exactly 20, 15, 5 or 0 cpd during a 5-day confinement period showed a dose-relationship between the number of cigarettes and the levels of urinary MHBMA (i.e., 6.3, 4.3, 2.2 and 0.5 µg/24h, respectively) (Theophilus 2015). A significant correlation between the number of cigarettes smoked per day and the levels of urinary MHBMA was shown in a European population, with an 11-fold increase in MHBMA in smokers vs. non-smokers, which correlated with the number of cpd (Lindner 2011).

Overall, smoking abstinence studies over periods of 3 to 8 days showed reductions of urinary levels of MHBMA of 6- to 30-fold, as compared to baseline levels, with an average of an around 10-fold reduction (Carmella 2012, Carmella 2009, Frost-Pineda 2008, Sarkar 2008, Theophilus 2015, Tricker 2012a, Tricker 2012b, Tricker 2012c, Tricker 2012d).

MHBMA is listed in the report by the Institute of Medicine on the "Scientific standards for studies on modified risk tobacco products" as being a representative biomarker of exposure (IOM (Institute of Medicine) 2012).

Further details on the metabolism of 1,3-butadiene are discussed in the 1,3-butadiene factsheet [Appendix A6.1.3.2; Factsheet 1_3 Butadiene].

6.1.3.1.4.3 Carbonyls

6.1.3.1.4.3.1 3-HPMA - Acrolein

IARC lists acrolein in the Group 3 agents, i.e., not classifiable as to its carcinogenicity to humans (IARC (International Agency for Research on Cancer) 1995). It has been classified as a respiratory (RT) and cardiovascular toxicant (CT) by the FDA (U.S. Department of Health and Human Services 2012c) and is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012). Furthermore, acrolein is one of the 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering, based on its very high index with respect to its properties as a toxicant with non-cancer response (targets: respiratory system and eyes) (WHO Study

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Group 2008). Its main adverse effect in humans is primarily irritation (U.S. Department of Health and Human Services 2007a).

Acrolein occurs naturally in foods, is formed during cooking (pyrolysis of fat) and is formed during the combustion of fossil fuels. It is found in engine exhausts, wood and tobacco smoke and is formed during the heating of cooking oils. Human exposure occurs from these sources during their production and use (IARC (International Agency for Research on Cancer) 1995). The ubiquitous occurrence of acrolein in the environment and its endogenous formation by lipid peroxidation needs to be considered when assessing changes in 3-HPMA in tobacco smoke-related exposure. The half-life of 3-HPMA has been described to be of 5-9 hrs (Gregg 2013).

Levels of 3-HPMA among non-smokers and smokers can overlap, but the differences between the groups have largely been described as statistically significant. Substantial variability of acrolein levels exists in different populations. A recent study by Park et al. (Park 2015) has shown that 3-HPMA urinary levels in 2,200 smokers of 5 different ethnicities (African Americans, native Hawaiians, whites, Latinos and Japanese Americans) were significantly different between the 5 groups.

After 3 days of smoking abstinence, levels of 3-HPMA were reported to decrease by more than 7-fold (Carmella 2007). Several other studies showed a 1.5-to 8-fold decrease in levels of 3-HPMA after 5 to 8 days of smoking abstinence (McRobbie 2015, Theophilus 2015, Tricker 2012a, Tricker 2012b, Tricker 2012c, Tricker 2012d). A smoking cessation study by Carmella et al. (Carmella 2009) showed that 3-HPMA levels decreased significantly by 78% in smokers who abstained for 4 weeks, thus confirming their earlier findings (Carmella 2007).

3-HPMA is listed in the report of the Institute of Medicine on the "Scientific standards for studies on modified risk tobacco products" as being a "representative exposure biomarker related to tobacco carcinogens and toxicants" (IOM (Institute of Medicine) 2012).

Further details on the metabolism of acrolein are discussed in the acrolein factsheet [Appendix A6.1.3.3; Factsheet Acrolein].

6.1.3.1.4.3.2 3-HMPMA - Crotonaldehyde

Crotonaldehyde has been classified by IARC as "not classifiable as to its carcinogenicity to humans" (Group 3) (IARC (International Agency for Research on Cancer) 1995). FDA has classified this aldehyde as a respiratory toxicant (U.S. Department of Health and Human Services 2012c), since it is irritating to the upper respiratory tract even at low concentrations and can cause pulmonary edema (U.S. Department of Health and Human Services 2014c). It is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), but not of the WHO-9 list of nine HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

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Crotonaldehyde is formed by combustion and the general population can be exposed to it by inhaling tobacco smoke, gasoline and diesel engine exhausts, and smoke from wood burning. People working with crotonaldehyde to manufacture other chemicals may be exposed to higher levels. Crotonaldehyde is produced endogenously and is found in many food products (National Center for Biotechnology Information (NCBI)).

In general, aldehydes are readily metabolized by either oxidation, reduction or conjugation. Conjugation of glutathione with crotonaldehyde, leads to the formation of 3-hydroxy-1-methylpropylmercapturic acid (3-HMPMA; also called 4-hydroxybut-2-yl mercapturic acid, HBMA) (IOM (Institute of Medicine) 2012, Yuan 2014)). About 6–15% of the applied dose of crotonaldehyde were found to be excreted as 3-HMPMA (WHO (World Health Organization) 2008). The half-life of 3-HMPMA is generally considered to be in the range of 5-9 h (Gregg 2013, Scherer 2006b, van Welie 1992).

Cigarette smokers excrete about 3-fold more 3-HMPMA, and only slightly elevated amounts of other metabolites, in their urine compared to non-smokers (Scherer 2007b). 3-HMPMA is the BoExp commonly used to assess exposure to crotonaldehyde. Other studies report up to 7- or 8-fold differences between smokers and non-smokers (Hecht 2014, IOM (Institute of Medicine) 2012, Zhang 2014).

In smoking abstinence studies, levels of 3-HMPMA decreased by 3- to 9-fold, after 3 to 8 days (Carmella 2012, Carmella 2009, Frost-Pineda 2008, Scherer 2007b, Theophilus 2015).

3-HMPMA (i.e., HBMA) is listed in the report of the Institute of Medicine on the "Scientific standards for studies on modified risk tobacco products" as being a "representative exposure biomarker related to tobacco carcinogens and toxicants" (IOM (Institute of Medicine) 2012).

Further details on the metabolism of crotonaldehyde are discussed in the crotonaldehyde factsheet [Appendix A6.1.3.4; Factsheet Crotonaldehyde].

6.1.3.1.4.4 Acid Derivatives

6.1.3.1.4.4.1 CEMA - Acrylonitrile

The current classification of acrylonitrile by the International Agency for Research on Cancer (IARC) is "possibly carcinogenic to humans" (Group 2B) (IARC (International Agency for Research on Cancer) 1999). The FDA has classified it as a carcinogenic and a respiratory toxicant (U.S. Department of Health and Human Services 2012c). Acrylonitrile is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), but not of the WHO-9 list of nine HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

A meta-analysis of epidemiological studies with workplace exposure to acrylonitrile suggests a 25% increase in lung cancer risk attributable to occupational exposure (Sponsiello-Wang 2006). The California Environmental Protection Agency suggests, after having compared yields in the smoke of 26 common brands of CC that acrylonitrile, together with acetaldehyde and 1,3-butadiene, is associated with the largest calculated

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cancer risks (Pankow 2007). For acrylonitrile, this, however, remains somewhat controversial, as stated in a review looking at the 4 most informative cohort studies that concluded that so far results do not support a causal relationship between acrylonitrile and all cancers or any specific type of cancer (Cole 2008).

Acrylonitrile is released into the environment primarily from its chemical production and plastic products industries (>95% in the sample country) (WHO (World Health Organization) 2002). There are no known natural sources (WHO (World Health Organization) 2002).

CEMA is commonly used as a BoExp to acrylonitrile (Gregg 2013, IOM (Institute of Medicine) 2012). In a controlled study in human volunteers, urinary metabolite data suggest that the elimination of acrylonitrile followed first-order kinetics, with a half-life of 7 to 9 hours (Jakubowski 1987). However, the kinetics of excretion of CEMA has been shown not to be uniform and may actually follow a 2-phase elimination (Jakubowski 1987). Therefore the half-life of CEMA may have been significantly underestimated (Gregg 2013). An analysis derived from a PMI study [ClinicalTrials.gov: NCT01465880] estimated a half-life exceeding 17h (data on file; available upon request).

Urinary excretion of CEMA has been shown to be consistently higher in smokers than in non-smokers. This difference varies from study to study, but ranges in non-smokers are usually below 2 ng/mL (Minet 2011, Schettgen 2012, Schettgen 2009, Xiaotao 2014) and increase to levels of more than 20 to 205 ng/mL in smokers (Ding 2009, Minet 2011, Schettgen 2012, Schettgen 2009, Xiaotao 2014). Smokers excreted mean levels of 187 or 184 µg/L of CEMA, while non-smokers only excreted 4.6 or 1.9 µg/L, in 2 studies reported respectively by Alwis et al. (Alwis 2012) and Schettgen et al. (Schettgen 2012). Another study reported a CEMA excretion of 71 µg/day (Jacob 2013). CEMA levels in smokers are significantly correlated with ISO tar yield, daily cigarette consumption, and other urinary BoExp (such as nicotine and cotinine exposure) (Alwis 2012, Minet 2011, Wu 2012).

Smoking abstinence studies of 5 to 8 days showed significantly reduced levels of CEMA in urine by about 7- to 10-fold (Scherer 2014, Scherer 2010).

Further details on the metabolism of acrylonitrile are discussed in the acrylonitrile factsheet [Appendix A6.1.3.5; Factsheet Acrylonitrile].

6.1.3.1.4.5 Epoxides

6.1.3.1.4.5.1 HEMA – Ethylene Oxide

In epidemiological studies of exposure to ethylene oxide, the most frequently reported association has been with lymphatic and hematopoietic cancers. There is limited evidence in humans for the carcinogenicity of ethylene oxide, but there is sufficient evidence in experimental animals for its carcinogenicity (U.S Department of Health and Human Services 2011). In making the overall evaluation, the IARC Working Group took into consideration all the supporting evidence, and classified ethylene oxide as “carcinogenic to humans” (Group 1) (IARC (International Agency for Research on Cancer) 2008b). Ethylene oxide is not part of the FDA-18 list (FDA (Food and Drug Administration)

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2012), nor of the WHO-9 list of nine HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

FDA has classified ethylene oxide as a carcinogenic, respiratory and reproductive or developmental toxicant (U.S. Department of Health and Human Services 2012c). Ethylene oxide is irritating to mucous membranes including those associated with the respiratory system (U.S. Department of Health and Human Services 1990). It is ubiquitous in the environment, arising from both natural and man-made sources (OECD (Organization for Economic Co-operation and Development) 2005a) with the major natural sources being emissions from vegetation of all types, where ethylene functions as a plant hormone. The main anthropogenic sources are from combustion of gas, fuel, coal and biomass. The highest exposure concentrations to humans are due to ethylene from car engines. Ethylene is also produced endogenously (Bolt 1996). A substantial part of the smoking-related increase in urinary HEMA levels might also come from ethylene, the amount of which in mainstream smoke is about 100 times higher than that of ethylene oxide (levels of 0.2-0.3 mg per cigarette vs ca. 10 µg/cigarette of ethylene oxide) (Persson 1988, Roemer 2012).

From occupational studies, the correlation between exposure to ethylene oxide and urinary levels of HEMA, as well as the apparent absence of accumulation of HEMA with the number of days at work, has suggested a half-life of less than 5h for HEMA (Haufroid 2007).

HEMA concentrations in urine of smokers are <2 - 3.5-fold higher compared with those of non-smokers (Alwis 2015, Calafat 1999, Ding 2009, Eckert 2011, Hou 2012a, Pluym 2015, Schettgen 2008b, Xiaotao 2014). Smoking abstinence studies of 3 to 8 days showed a 3- to 4-fold decrease of HEMA levels (Carmella 2012, Carmella 2009, Scherer 2010, Theophilus 2015). Because exposure of the general population to ethylene, ethylene oxide and acrylonitrile comes mainly from cigarette smoke, HEMA remains a suitable BoExp for ethylene oxide exposure, despite its lack of specificity to either one of these 3 compounds.

HEMA is listed in the report of the Institute of Medicine on the "Scientific standards for studies on modified risk tobacco products" as being a "representative exposure biomarker related to tobacco carcinogens and toxicants" (IOM (Institute of Medicine) 2012).

Further details on the metabolism of ethylene oxide are discussed in the ethylene oxide factsheet [Appendix A6.1.3.6; Factsheet Ethylene Oxide].

6.1.3.1.4.6 Monocyclic Aromatic Hydrocarbons

6.1.3.1.4.6.1 S-PMA – Benzene

Benzene has been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC) since the late 1980's (Group 1; carcinogenic to humans) (IARC (International Agency for Research on Cancer) 2012b). With respect to cancer hazards, benzene ranks amongst the highest of HPHCs contained in cigarette smoke (WHO Study Group 2008). It is one of the 9 HPHCs suggested by the WHO Working

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Group on Tobacco Regulation for mandated lowering in tobacco smoke (WHO Study Group 2008) and is also part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012). FDA has classified benzene as a carcinogen, and as a cardiovascular and reproductive or developmental toxicant (U.S. Department of Health and Human Services 2012c). Cigarette smoking was reported to be causally related to myeloid leukemia and benzene has been associated with the estimated excess of acute myeloid leukemia (IARC (International Agency for Research on Cancer) 2004)

There are a number of possible sources of benzene intake, other than cigarette smoke, including industrial emissions, automobile exhaust fumes, glues, solvents and paints (U.S. Department of Health and Human Services 2007b). Natural sources include oil seeps and forest fires. Individuals may also be exposed to benzene by consuming contaminated water.

Benzene metabolism is well described, and several metabolites can be measured as BoExp (Arnold 2013). Phenol, the main metabolite, is formed from the initial oxidation product benzene oxide. Phenol is rapidly oxidized to catechol or hydroquinone, which can be further oxidized to reactive benzoquinones. Each of the phenolic metabolites of benzene, such as phenol, catechol and hydroquinone, can undergo sulfonic or glucuronic conjugation resulting in conjugates that are major urinary metabolites of benzene. Other pathways of benzene oxide metabolism include: 1) reaction with glutathione (GSH) to form S-phenylmercapturic acid (S-PMA), and 2) iron-catalyzed ring-opening conversion to trans,trans-muconic acid (tt-MA).

Most metabolites of benzene are either non-specific for benzene or lack sensitive analytical methods (Boogaard 1996, Medeiros 1997, Ruppert 1997). Phenol is not a suitable BoExp for benzene exposure as the background levels are highly variable due to endogenous production of phenol as well as dietary and environmental exposure. Also, as both catechol and hydroquinone are smoke constituents themselves, both have to be considered as unspecific and unreliable indicators of exposure to benzene. tt-MA generally shows a good correlation with medium to high levels of benzene exposure, but has also been found in subjects not exposed to benzene. Consequently, the levels of tt-MA in the urine of smokers and non-smokers were found to be not statistically different (Lovreglio 2010). Assessment of tt-MA as a BoExp for benzene thus remains controversial. Finally, sorbic acid (2,4-hexadienoic acid), a food additive that can be transformed metabolically to tt-MA, can contribute to background levels of tt-MA, thereby decreasing its specificity as a BoExp to benzene in levels relevant for smoking (Pezzagno 1999, Scherer 1998).

S-PMA in urine is currently the most specific and widely used BoExp to benzene (Fustinoni 2005) and appears to be the most suitable BoExp to assess exposure of smokers to benzene contained in cigarette smoke. S-PMA's half-life is around 9 hours (Benowitz 2009, Boogaard 1996, van Sittert 1993).

Levels of the urinary metabolite of benzene, S-PMA, were reported to be higher in smokers than in non-smokers (Boogaard 1995, Boogaard 1996, Lindner 2011, Schettgen 2010).-After smoking abstinence of 3 to 8 days, levels of urinary S-PMA were found to decrease by about 2-fold up to more than 20-fold. In a smoking abstinence study over 8

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days, S-PMA was found to be more sensitive than tt-MA (Feng 2006). S-PMA decreased by around 20 fold (6.52 to 0.32 µg/24h) and tt-MA by about 2-fold (0.25 to 0.13 mg/24h). In another smoking abstinence study, S-PMA decreased by about 8-fold and 12-fold after 3 and 7 days of cessation, respectively (Carmella 2009). Benzene is listed in the report of the Institute of Medicine on the "Scientific standards for studies on modified risk tobacco products" as being a representative BoExp (IOM (Institute of Medicine) 2012).

Further details on the metabolism of benzene are discussed in the benzene factsheet [Appendix A6.1.3.7; Factsheet Benzene].

6.1.3.1.4.6.2 S-BMA - Toluene

IARC has classified toluene as "not classifiable as to its carcinogenicity to humans" (Group 3) (IARC (International Agency for Research on Cancer) 1999) considering the weak consistency of findings in 8 studies evaluating association between exposure to toluene and cancer. Toluene is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), but not of the WHO-9 list of nine HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

FDA reports it as a respiratory and reproductive or developmental toxicant (U.S. Department of Health and Human Services 2012c). In humans, respiratory tract irritation is experienced from exposure to toluene, and irritation of the upper airways (U.S. Department of Health and Human Services 2000). Reproductive toxicity has been observed in exposed humans (spontaneous abortion) (IARC (International Agency for Research on Cancer) 1999).

Toluene is released into the atmosphere principally from the volatilization of petroleum fuels, toluene-based solvents and thinners as well as from motor vehicle exhausts. It has also been detected in emissions from forest fires, volcanos and crude oil (National Center for Biotechnology Information (NCBI) , U.S. Department of Health and Human Services 2000) .

Much of the toluene inhaled is exhaled unchanged. The primary initial steps in toluene metabolism from the absorbed toluene leads to the formation of benzyl alcohol and benzoic acid (U.S. Department of Health and Human Services 2000), which is further metabolized to hippuric acid (U.S. Department of Health and Human Services 2000). S-benzyl mercapturic acid (S-BMA), a minor urinary metabolite of toluene identified in humans, is thought to be formed via conjugation of benzyl alcohol with glutathione. In humans, up to about 75–80% of inhaled toluene that is absorbed can be accounted for as hippuric acid in the urine (U.S. Department of Health and Human Services 2000). S-BMA has been considered as having a greater validity to evaluate exposure to toluene than hippuric acid, as there are a number of hippuric acid precursors present in the environment (e.g., benzoic acid in food) (Inoue 2004, Lovreglio 2010, U.S. Department of Health and Human Services 2000).

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The excretion of toluene and its metabolites is rapid, with the major portion occurring within 12 hrs of exposure. No information is available regarding the *in vivo* conversion from toluene to S-BMA. It is possible, however, to estimate that the half-life for S-BMA in men would be no more than 10 hrs, by analogy to the half-life of 9 hrs for S-PMA excretion after occupational exposure to benzene (Inoue 2004).

In studies which have considered smokers and non-smokers, determination of S-BMA could not differentiate between smokers and non-smokers, but was sufficient for determination of occupational toluene exposure (B'Hymer 2011, Imbriani 1999, Lovreglio 2010, Maestri 1997, Pluym 2015). A study conducted in Germany using HPLC-MS/MS reports median levels of 8.2 (range: 1.6-77.4 µg/L) and 11.5 (range: 0.9-51.2 µg/L) in non-smokers and smokers, respectively, the difference being not significantly different (Schettgen 2008a).

The reason for the difficulty to discriminate smokers from non-smokers based on the urinary levels of S-BMA has not been clarified, but it was hypothesized 1) that other environmental sources of toluene are contributing in such a way to the individual exposure that smoking is only a minor factor or 2) that the specificity of S-BMA excretion as a biomarker of toluene exposure is insufficient for the determination of environmental exposures and possibly other metabolic routes might in part also lead to the formation (and excretion) of S-BMA (Schettgen 2008a). Benzyl alcohol, which is a widespread constituent of cosmetic products like shampoo, creams, after-shave lotions or fragrances, could largely influence the excretion of S-BMA, as it is part of the same metabolic pathway as toluene (Imbriani 1999, Lovreglio 2010). The presence of substantial background levels of S-BMA in the general population with no occupational exposure to toluene has been observed even in the absence of cigarette smoking (Munaka 2009, Pluym 2015).

Toluene is part of the FDA-18 list of HPHCs to report, PMI included it with S-BMA as a BoExp, in the clinical assessment program for THS, despite the considerable uncertainty on the suitability of S-BMA to indicate exposure to tobacco smoke.

Further details on the metabolism of toluene are discussed in the toluene factsheet [Appendix A6.1.3.8; Factsheet Toluene].

6.1.3.1.4.7 Aromatic Amines

6.1.3.1.4.7.1 1-NA

The current classification of 1-aminonaphthalene (1-NA) by the International Agency for Research on Cancer (IARC) is “inadequate evidence of carcinogenicity” (Group 3) (IARC (International Agency for Research on Cancer) 1998), while the FDA has classified it as carcinogenic (U.S. Department of Health and Human Services 2012c). It is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), but not of the WHO-9 list of nine HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008). An excess occurrence of bladder cancer was observed in workers who had been exposed to commercial 1-NA for 5 or more years (International Programme on Chemical Safety (IPCS) 1987). In several studies, it has not been possible to single out 1-NA from

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the mixed exposures investigated, rendering a conclusive assessment on its carcinogenicity in humans difficult.

No information could be found in the literature on the half-life of 1-NA.

Only very little data are available on levels of 1-NA in the urine of smokers and non-smokers. Grimmer et al. (Grimmer 2000) reported a difference of urinary excretion of 1 NA between smokers and non-smokers of about 7-fold, and Yu et al. of about 5-fold (Yu 2014).

No studies investigating the effect of smoking abstinence/cessation are, to our knowledge, available to date.

Further details on the metabolism of 1-NA are discussed in the 1-NA factsheet [Appendix A6.1.3.9; Factsheet 1-NA].

6.1.3.1.4.7.2 2-NA

The classification of 2-aminonaphthalene (2-NA) by both the International Agency for Research on Cancer (IARC) and FDA is "carcinogenic to humans" (IARC (International Agency for Research on Cancer) 2010b, U.S. Department of Health and Human Services 2012c). 2-NA is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), but not of the WHO-9 list of the nine HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008). Several epidemiological studies have shown a correlation between exposure to 2-NA and increase of bladder cancer incidence (U.S. Department of Health and Human Services 2014b). In the last century, up to 100% of individuals involved in the purification of 2-NA for use in the dye industry developed bladder cancer (National Center for Biotechnology Information (NCBI) 2014).

2-NA is formed during the pyrolysis of nitrogen-containing organic matter and can occur as such in nature. Occupational exposure and general population exposure should be low or non-existent since 2-naphthylamine is no longer produced or used (it was previously produced for the manufacture of rubber and dyes). The general population can be exposed to 2-naphthylamine through the inhalation of tobacco smoke, as reported by IARC (IARC (International Agency for Research on Cancer) 2010b).

A total of 24 metabolites of 2-NA have been identified (IARC (International Agency for Research on Cancer) 2010b) in laboratory animal studies. Metabolism occurs via 3 main pathways: 1) N-hydroxylation; 2) conjugation of the amino group with acetic, sulfuric, or glucuronic acid; 3) oxidation at C5 and C6 to an arene oxide. The proportion of these metabolites excreted in the urine of various laboratory animals is different, although in most species 2-amino-1-naphthyl sulfate is the predominant metabolite. The pathways involved in the formation of reactive intermediates via oxidation of the amine moiety have been associated with carcinogenesis.

Because of the known carcinogenicity of 2-NA, few metabolic studies have been carried out in humans. The analysis of the parent aromatic amines in urine has often been used to biomonitor exposure to these compounds. Mostly, this has been performed after hydrolysis of the urine thus encompassing both conjugated and free aromatic amines.

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No information could be found in the literature on the half-life of 2-NA.

Lindner et al. have shown a very good relationship between the urinary excretion of nicotine and its major metabolites (summarized as nicotine equivalents) and 2-NA (Lindner 2011). The difference in urinary 2-NA levels in smokers and non-smokers range from less than 2-fold (Grimmer 2000, Riedel 2006, Riffelmann 1995) to up to 10-fold (Lindner 2011). A recent study in which smokers consuming 20 cpd or more were forced to smoke exactly 20, 15, 5 or 0 cpd during a 5-day confinement period showed a dose-relationship between the number of cigarettes and the levels of urinary 2-NA (i.e., 31.2, 21.6, 12.1 and 3.6 ng/24h, respectively) (Theophilus 2015). It was also shown that 2-NA urinary levels increased with tar yield, and also that they are strongly associated with cigarette consumption, increasing with higher consumption (Lindner 2011).

2-NA has also been investigated in several studies in which switching from conventional cigarettes to smoking abstinence was demanded (Tricker 2012a, Tricker 2012b, Tricker 2012c, Tricker 2012d). Within 8 days, the urinary excretion of 2-NA dropped by 6- to 7-fold to reach non-smokers' levels (Frost-Pineda 2008, Tricker 2012a). All studies reported 2-NA excretion in non-smokers and relatively broad variance within groups. The origin of 2-NA in the urine of current non-smokers is unknown. A possible source might be 2-nitronaphthaline, which may be generated by the combustion or pyrolysis of organic material, such as during cooking or in engine exhaust (Heeb 2010).

2-NA is known to form hemoglobin adducts, which were found to be statistically significantly higher in smokers compared to non-smokers (Bryant 1988). However, 2-NA hemoglobin adducts were rarely used so far for biomonitoring, probably because of methodological challenges.

Further details on the metabolism of 2-NA are discussed in the 2-NA factsheet [Appendix A6.1.3.10; Factsheet 2-NA].

6.1.3.1.4.7.3 4-ABP

4-Aminobiphenyl (4-ABP) has been classified as carcinogenic to humans by IARC some decades ago (Group 1; carcinogenic to humans) (IARC (International Agency for Research on Cancer) 2010c). It was placed on the initial list of priority toxicants suggested by the WHO Working Group on Tobacco Regulation (i.e., WHO-18 (WHO Study Group 2008)), however, because the experimental data on 4-ABP do not allow proper calculation of a T25 value (i.e., the long-term daily dose that will produce tumors in 25% of animals above the background rate at a specific tissue site), it was not added to the final WHO-9 list of 9 HPHCs recommended for mandated lowering in cigarette smoke (WHO Study Group 2008). The FDA has also classified 4-ABP as carcinogen (U.S. Department of Health and Human Services 2012c), as many case reports and cohort-surveillance studies indicate a high occurrence of cancer of the urinary bladder in workers occupationally exposed to 4-ABP (IARC (International Agency for Research on Cancer) 2012b).

Because of its carcinogenic effects, likely due to metabolic activation of 4-ABP to reactive intermediates (leading to DNA- or hemoglobin adducts), production and use of 4-ABP has been prohibited since many years (IARC (International Agency for Research

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on Cancer) 2010c). Cigarette smoke is thus the main source of exposure for the general population, but exposure may also occur through hair dyes, food colorant, fungicide used on apples, fumes from cooking oils, diesel exhausts and benzidine-contaminated sites. 4-ABP is not known to occur in nature.

From animal studies, the half-life of urinary 4-ABP has been estimated to be between 15 and 30 hours (Karreth 1991). Based on PMI clinical studies, the half-life of urinary 4-ABP is estimated within the same range in humans (unpublished PMI data).

A recent study in which smokers consuming 20 cpd or more were forced to smoke exactly 20, 15, 5 or 0 cpd during a 5-day confinement period showed a dose-relationship between the number of cigarettes and the levels of urinary 4-ABP (i.e., 22.4, 16.6, 10.9, and 5.5 ng/24h, respectively) (Theophilus 2015). Other studies reported urinary 4-ABP levels in smokers to be 3 to 9-fold higher than in non-smokers (Lindner 2011, Miura 2015, Roethig 2009, Scherer 2014, Seyler 2011, Yu 2014). A moderate correlation between the urinary excretion of 4-ABP in smokers and the extent of smoking, measured by daily cigarette consumption, was found (Lindner 2011). Few studies (Grimmer et al. (Grimmer 2000) and Riedel et al. (Riedel 2006)) found no or only small differences in urinary 4-ABP concentrations between smokers and non-smokers (1.1 and 1.6 fold difference, respectively). The inconsistency in findings in the urinary 4-ABP levels between smokers and non-smokers might be partially explained by the different sensitivities of the methods used for the analyses.

In 6 studies with smoking abstinence groups, the change of urinary excretion of 4-ABP was about 2- to 5-fold after 5 or 8 days of smoking abstinence, which is consistent with the order of magnitude for the difference observed between smokers and non-smokers (Frost-Pineda 2008, Sakaguchi 2014, Theophilus 2015, Tricker 2012a, Tricker 2012b, Tricker 2012c). Sarkar et al. (Sarkar 2010) reported higher differences (about 8-fold) in urinary 4-ABP levels after 8 days in confinement, both for active smokers who abstained from smoking.

Further details on the metabolism of 4-ABP are discussed in the 4-ABP factsheet [Appendix A6.1.3.11; Factsheet 4-ABP].

6.1.3.1.4.7.4 o-Toluidine

The classification of *ortho*-toluidine (*o*-toluidine) by both the International Agency for Research on Cancer (IARC) and FDA is “carcinogenic to humans” (IARC (International Agency for Research on Cancer) 2012b, IARC (International Agency for Research on Cancer) 2010b, U.S. Department of Health and Human Services 2012c). Overall, epidemiological studies show consistent associations between exposure to *ortho*-toluidine and bladder cancer (IARC (International Agency for Research on Cancer) 2012b, U.S. Department of Health and Human Services). *o*-Toluidine is not part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), nor of the WHO-9 list of 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

The general population is known to be exposed ubiquitously to *o*-toluidine (IARC (International Agency for Research on Cancer) 2010a, Richter 2015, WHO (World

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Health Organization) 1998). *o*-Toluidine is detected in µg/kg amounts in meat and dairy products and in upper ng/kg amounts in salads, vegetables, eggs, alcoholic beverages, cereals and fish. Even higher concentrations in the upper µg/kg range were reported for ice cream powders, food colorings and soft drink concentrates. *o*-Toluidine occurs in tobacco leaves and in the aroma of black tea and is also present in ambient air in ng/m^3 concentrations (Tricker 2009).

No data on the half-life of *o*-toluidine after systemic exposure exists in the literature. After topical application of an anesthetic gel containing *o*-toluidine to humans, the half-life of *o*-toluidine was reported to be 4 hours in the plasma (Herdevall 2003).

Tobacco smoke as a source of human exposure to *o*-toluidine was first shown by El-Bayoumy et al. (el-Bayoumy 1986) who reported a 1.5-fold higher excretion of free *o*-toluidine in smokers compared to non-smokers. This was confirmed in a series of other studies. The differences between smokers and non-smokers range between 1.3- and 1.8-fold (el-Bayoumy 1986, Kutting 2009, Lindner 2011, Riedel 2006, Riffelmann 1995, Shepperd 2013, Weiss 2005). A clear relationship between the amount of arylamines, including *o*-toluidine, excreted in urine and the measures of smoking dose (i.e., self-reported cigarette per day, exhaled CO, cotinine in saliva, and nicotine equivalents in urine) has been shown by Riedel et al. (Riedel 2006). Urinary levels of *o*-toluidine were found to increase with tar yields, and to be associated with cigarette consumption, increasing with higher consumption; whereas, no association between *o*-toluidine levels and gender or age was found (Lindner 2011).

In 5 confined 8-days studies, urinary levels of *o*-toluidine decreased from 2- to 3.6-fold in abstinent smokers (Frost-Pineda 2008, Sarkar 2010, Tricker 2012a, Tricker 2012b, Tricker 2012d). In a 5-day study, this decrease was found to be 2.7 fold (Tricker 2012c).

However, other environmental sources of *o*-toluidine play an important role in total *o*-toluidine exposure, leading to reports in the literature with non-smokers having even higher levels of urinary *o*-toluidine than smokers in e.g. one occupational study (1.4-fold) (Ward 1996).

Despite the fact that the general population is known to be exposed ubiquitously to *o*-toluidine, PMI decided to include *o*-toluidine in the assessment program of THS due to its IARC classification.

Further details on the metabolism of *o*-toluidine are discussed in the o-toluidine factsheet [Appendix A6.1.3.12; Factsheet *o*-Toluidine].

6.1.3.1.4.8 Tobacco Specific Nitrosamines

6.1.3.1.4.8.1 Total NNAL and Total NNN – NNK and NNN

Of the described tobacco-specific N-nitrosamines (TSNAs), both 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) have been classified by the IARC as being carcinogenic to humans (Group 1) (IARC (International Agency for Research on Cancer) 2012a). FDA has also classified them as carcinogenic (U.S. Department of Health and Human Services 2012c) and they are 2 of the 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in

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cigarette smoke (WHO Study Group 2008). They are also part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012). Epidemiological studies of tobacco users, both smoked and smokeless products, support a plausible association between exposure to NNN and NNK and cancer (IARC (International Agency for Research on Cancer) 2007) and may indicate an association of NNK metabolites (i.e. total NNAL) with lung cancer in smokers. It is, however, impossible to exclude the effect of other carcinogens present in tobacco smoke (IARC (International Agency for Research on Cancer) 2012a). However, NNK has recently been reported to be associated with lung cancer risk, independently of smoking intensity and duration (Yuan 2014).

TSNAs, including NNK, NNN, N'-nitrosoanabasine (NAB) and N'-nitrosoanatabine (NAT), occur widely in tobacco and tobacco smoke (Toxicology Data Network 2010). They are formed by the nitrosation of nicotine and other tobacco alkaloids and have occasionally been detected in green tobacco leaves; however, the largest quantities of tobacco-specific N-nitrosamines are formed during tobacco curing and processing and additional amounts may be formed during smoking by pyrosynthesis. TSNAs occur in all tobacco products including smokeless tobacco-containing products (Toxicology Data Network 2010). Because most of the TSNA yield in cigarette smoke comes from unchanged transfer from the tobacco by distillation with little contribution by pyrolysis (Fischer 1990, Moldoveanu 2008), their yields are dependent mainly on their respective content in the cigarette tobacco blend. However, as a recent study by Forster et al. (Forster 2015) demonstrated, a certain temperature is needed to release TNSAs from the tobacco. The evaporative transfer of NNK and NNN present in cured tobacco to the mainstream aerosol occurred at low temperatures but to the most extent above 120°C to 140°C, although no clear linearity could be shown in the temperature range assessed in this study. Furthermore, the overall percentage of TSNAs released at the temperatures assessed in this study (100°C to 200°C) was very low (<10%) compared to the levels of TSNAs contained in the tobacco used. This is important to be considered for the assessment of heat-not-burn products, such as THS, and makes the TSNAs, besides their specificity to tobacco, a valuable marker to assess the effectiveness of a heat-not-burn product.

To assess exposure to NNK, the urinary level of total NNAL is usually determined, i.e., the molar sum of NNAL plus both its O- and N-glucuronide conjugates. NNK itself is rapidly metabolized, so that it is usually not detectable in the urine of smokers (Hu 2014). For NNN, excretion of total NNN, i.e., NNN plus its pyridine N-glucuronide, is usually measured.

TSNAs are optimally suited for biomonitoring tobacco exposure because of their specificity to tobacco. Total NNAL has been used in studies on smoking behavior (Le Marchand 2008), cessation (Carmella 2009), or switching to alternative products (Hatsukami 2004). Baseline levels of excreted total NNAL (NNAL and NNAL-Gluc) in the 24-h urine of smokers, are typically in the range of 1.1-2.9 nmol/24h (i.e., 230-607 ng/24h) (IOM (Institute of Medicine) 2012). For total NNN, the excretion levels are in the range of 0.049-0.24 nmol/24h (i.e., 8.7-42.5 ng/24h) (IOM (Institute of Medicine) 2012). Neither total NNAL nor total NNN are, in principle, detectable in the urine of non-smokers, unless they use other tobacco products, nicotine replacement products, or are

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exposed to secondhand smoke, in which case levels are usually less than 5 percent of smoker levels (IARC (International Agency for Research on Cancer) 2007, IOM (Institute of Medicine) 2012).

Because of the long half-life of NNAL, estimated to be between 10 and up to 45 days (Goniewicz 2009, Hecht 1999b), with the commonly referenced half-life being between 10-18 days, total NNAL levels in smoking abstinence studies of short duration do not reach the levels of non-smokers and decrease by around 3- to 4-fold in studies lasting between 3 to 8 days (Carmella 2009, Roethig 2007, Tricker 2012a, Tricker 2012b, Tricker 2012c, Tricker 2012d). In a smoking abstinence study lasting up to 56 days, levels of total NNAL reached levels described for non-smokers at the end of the study (i.e., 0.13 nmol/24h at day 56 vs 2.70 nmol/24h at baseline), and seemed to remain above these levels after 28 and 42 days (i.e., 0.26 and 0.2 nmol/24h, respectively) (Carmella 2009). Total urinary levels of NNN have not been as extensively studied as total NNAL. One study showed that the levels decrease below the quantification levels after 5 days (Urban 2009). In a smoking abstinence study with nicotine replacement therapy (NRT) use, total NNN levels decreased by 89% vs baseline after 3 days (Stepanov 2009a), whereas it took an average of 4 weeks for total NNAL to decrease to the same level.

Both NNAL and NNN are listed in the report of the Institute of Medicine on the "Scientific standards for studies on modified risk tobacco products" as being "representative exposure biomarkers related to tobacco carcinogens and toxicants" (IOM (Institute of Medicine) 2012).

Further details on the metabolism of NNK and NNN are discussed in the NNK and NNN factsheets [Appendix A6.1.3.13; Factsheet NNK, and Appendix A6.1.3.14; Factsheet NNN].

6.1.3.1.4.9 Polycyclic Aromatic Hydrocarbons

6.1.3.1.4.9.1 Total 3-OH-B[a]P - Benzo[a]pyrene

Benzo[a]pyrene (B[a]P) is classified as "carcinogenic to humans" by IARC (Group 1) (IARC (International Agency for Research on Cancer) 2012c) and by FDA (U.S. Department of Health and Human Services 2012c). It is used by the WHO as evidence supporting an important role for PAHs as causes of lung and other cancers in smokers (Burns 2008, WHO Study Group 2008). No epidemiological data on benzo[a]pyrene alone are available, however, there is sufficient evidence for the carcinogenicity of B[a]P in experimental animals (IARC (International Agency for Research on Cancer) 2012c). In humans, occupational exposures to B[a]P-containing mixtures have been associated with a series of cancers: coke production: lung; coal gasification: lung, bladder; paving and roofing: lung; coal tar distillation: skin; soots: lung, esophagus, hematolymphatic system, skin; aluminum smelting: lung, bladder; tobacco smoking: lung, lip, oral cavity, pharynx, esophagus, larynx, bladder (IARC (International Agency for Research on Cancer) 2012c). B[a]P is one of the 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008), and is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012).

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B[a]P and other PAHs are widespread environmental contaminants formed during incomplete combustion or pyrolysis of organic material (IARC (International Agency for Research on Cancer) 2012c, U.S. Department of Health and Human Services 1995). Major sources of PAHs in ambient air (both outdoors and indoors) include residential and commercial heating with wood, coal or other biomass fuels (oil and gas heating produce much lower quantities of PAH), other indoor sources such as cooking and tobacco smoke, and outdoor sources including motor-vehicle exhaust (especially from diesel engines), industrial emissions and forest fires.

Several metabolites of B[a]P can induce mutations, transform cells and/or bind to cellular macromolecules; however only the 7,8-diol-9,10-epoxide is presently considered to be an ultimate carcinogenic metabolite. Total 3-hydroxybenzo[a]pyrene (3-OH-B[a]P, free or sulphate and glucuronide conjugates) represents about 40% of the metabolite fraction (Ball 1979, Yang 1975), and is used as the BoExp to B[a]P in PMI clinical studies.

The half-life of B[a]P in human urine was estimated at 3-4 hrs (Chien 2012).

The validation of fit-to-purpose of both 1-OHP, a surrogate marker for PAHs, and total 3-OH-B[a]P remains equivocal (Gregg 2013). An approximately 2-fold difference between smokers and non-smokers was observed in some studies (Lindner 2011, Sarkar 2010), but the ranges of values for smokers and non-smokers overlapped in other studies (Gregg 2013, Lafontaine 2006, Scherer 2007a, Suwan-ampai 2009).

A smoking abstinence for 5 days showed reduction of urinary total 3-OH-B[a]P levels by 3.5-fold (Theophilus 2015). Furthermore, a reduction by about 3-fold of total 3-OH-B[a]P levels was observed when switching from conventional cigarettes to snus oral tobacco consumption or after smoking abstinence for 8 days (Sarkar 2010). However, long-term data comparisons in smoking abstinence studies and other properties like urinary accumulation kinetics were not found.

Further details on the metabolism of benzo[a]pyrene are discussed in the benzo[a]pyrene factsheet [Appendix A6.1.3.15; Factsheet BaP].

6.1.3.1.4.9.2 Total 1-OHP - Pyrene

Pyrene is not present on any of the HPHC lists, as it is considered non-carcinogenic. However, due to its abundance in any mixture of polycyclic aromatic hydrocarbons (PAHs), its metabolite 1-hydroxypyrene (1-OHP) has often been used to monitor exposure to PAHs, several of which are carcinogenic (Brandt 2003, Jongeneelen 1988, Roethig 2009, Strickland 1996).

Pyrene occurs ubiquitously in products of incomplete combustion, in fossil fuels (i.e., mineral oils, petrol, etc.) and is found in relatively high quantities in coal tar. Broiled and smoked foods are also sources of pyrene exposure (Strickland 1999).

Pyrene is primarily (~ 90%) metabolized to 1-hydroxypyrene (1-OHP) by CYP1A enzymes and is renally eliminated as glucuronide and sulfate conjugates ('total 1-OHP') (Brandt 2003). The conjugate of 1-OHP with glucuronide is the predominant form of pyrene metabolite in urine (Hu 2006). 1-OHP in urine is generally assessed together with

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its glucuronide and sulfate conjugates (i.e., total 1-OHP). The urinary half-life of urinary 1-OHP excretion in humans ranges from 4 to 35 h (Jongeneelen 1988).

Pyrene is one of the PAHs found in cigarette smoke and is often used as an indicator/surrogate of total PAH exposure, although not carcinogenic (Roethig 2009). Total 1-OHP is generally considered an appropriate BoExp to toxicologically relevant PAHs, such as benzo[a]pyrene, as it is rapidly distributed, metabolized and eliminated from the body (Bouchard 1998). In addition, 1-OHP in urine represents a constant fraction (~2%) of total pyrene intake (Jongeneelen 1988). 1-OHP is listed in the report of the Institute of Medicine on the "Scientific standards for studies on modified risk tobacco products" as being a "representative exposure biomarker related to tobacco carcinogens and toxicants" (IOM (Institute of Medicine) 2012).

A substantial variability in biologic response to PAHs is expected because of inter-individual differences (genetic polymorphisms) in the activity of the enzyme systems involved in their metabolism (Alexandrie 2000, MacLeod 1997). In smokers, 2- to 5-fold higher levels of urinary total 1-OHP were reported in numerous studies compared to non-smokers (Alexandrie 2000, Barbeau 2011, Buratti 2007, Dor 2000, Elmstahl 1991, IOM (Institute of Medicine) 2012, Jacob 1999, Jongeneelen 1988, Kim 2001, Li 2000, Lindner 2011, Melikian 2007, Mendes 2008, Merlo 1998, Morin 2011, Nerurkar 2000, Pastorelli 1999, Roethig 2009, Roggi 1997, Scherer 2007a, Scherer 2000, Shepperd 2013, Sithisarankul 1997, St Helen 2012, Szaniszlo 2001, van Delft 2001).

In smoking abstinence studies, the levels of urinary 1-OHP decreased by <2 - 4-fold after 5 to 8 days of smoking cessation (Carmella 2012, Carmella 2009, Feng 2006, Frost-Pineda 2008, Martin Leroy 2012, Theophilus 2015, Tricker 2012a, Tricker 2012b, Tricker 2012c, Tricker 2012d).

Further details on the metabolism of pyrene are discussed in the pyrene factsheet [Appendix A6.1.3.16; Factsheet Pyrene].

6.1.3.1.4.10 Alkaloids

6.1.3.1.4.10.1 Nicotine and its Metabolites

Nicotine has been classified by the FDA as an addictive and reproductive or developmental toxicant (U.S. Department of Health and Human Services 2012c). Addiction develops as a neurobiologic adaptation to chronic nicotine exposure (U.S. Department of Health and Human Services 2014a). The reasons for nicotine use are enhancement of mood, either directly or through relief of withdrawal symptoms, and augmentation of mental or physical functions (Benowitz 2010). The pharmacological basis of the addictiveness of nicotine can be seen as a combination of positive reinforcements, e.g. response to a negative affection such as craving and withdrawal symptoms (Benowitz 2008). Nicotine has been identified as a potential cause of reduced fertility as well as maternal, fetal, and infant morbidity and mortality (Mitchell 2006). Animal studies with nicotine have shown adverse effects on the development of the central nervous system and nicotine effects on the brain have been postulated to be involved in the pathophysiology of Sudden Infant Death Syndrome (Mitchell 2006). Nicotine is part of the of the FDA-18 HPHCs to report (FDA (Food and Drug

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Administration) 2012), but not of the WHO-9 list of 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

Nicotine is the principal alkaloid (about 95% of the total alkaloid content) found in tobacco (Hukkanen 2005) and starts to transfer into the smoke at temperatures below 300°C (Baker 1987). Seventy percent of the nicotine in tobacco is recovered intact after smoking. The remaining 30% is converted to other substances (ending up mostly in the sidestream smoke) (Houseman 1973, Philip Morris USA 2001). Nicotine is most frequently encountered in tobacco products, used for smoking, chewing or snuffing but also found in low levels in vegetables such as potatoes, tomatoes, and eggplants (Domino 1993, Siegmund 1999). It is used as an adjunct to smoking cessation programs (gums, patches, inhalers), so called nicotine replacement therapies (NRT) (International Programme on Chemical Safety (IPCS)).

Nicotine can be extremely toxic and has been largely considered as being lethal at oral doses of 60 mg (for an adult) (Toxicology Data Network). However, recent review of available data in the literature on nicotine suggest that the lethal dose is more than 10-fold higher than the largely cited 60 mg dose (Mayer 2014).

The plasma half-life of nicotine is around 2hrs (Benowitz 2009) in populations with normal CYP2A6 activity, but can be considerably longer if CYP2A6 activity is impaired. When the half-life of nicotine is determined using the time course of urinary excretion, its terminal half-life averages 11hrs (Benowitz 2009). The half-life of Cotinine, the main metabolite of Nicotine, is much longer than the half-life of nicotine at approximately 16h (Benowitz 2009). Cotinine concentrations in plasma are linearly and directly related to nicotine intake and show little variation for up to 2hrs after the last exposure to nicotine (Galeazzi 1985), although individual variability in percentage conversion of nicotine to cotinine and in rate of elimination of cotinine itself exists (Benowitz 2009).
Because of the almost exclusive occurrence of nicotine in larger amounts in tobacco or NRT products, the presence of its metabolite cotinine in plasma or urine is used to confirm the smoking status (or NRT use) of a person.

Nicotine and its 5 major metabolites (NEQ) are listed in the report of the Institute of Medicine on the “Scientific standards for studies on modified risk tobacco products” as being a “representative exposure biomarker related to tobacco carcinogens and toxicants” (IOM (Institute of Medicine) 2012). NEQ accounts for 85%–90% of the systemic dose of nicotine absorbed, as assessed from transdermal nicotine application (Benowitz 2011, Benowitz 1994).

In smokers, blood or plasma nicotine concentrations during the day typically range between 10 and 37 ng/mL, with peak concentrations between 19 and 50 ng/mL (Benowitz 2009). Urinary concentrations of NEQ range from 70.4 to 154 µmol/24h (i.e., 11.42-24.98 mg/24h) (IOM (Institute of Medicine) 2012). Low levels (i.e., 0.00 to 2.21 mg/24h) were achieved after 5 to 8 days of smoking cessation (Frost-Pineda 2008, Roethig 2007, Theophilus 2015, Tricker 2012a, Tricker 2012b, Tricker 2012c, Tricker 2012d).

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However, as discussed by Gregg et al. (Gregg 2013), nicotine is not fit-for-purpose as a BoExp in studies on candidate MRTPs such as THS which aims to approach nicotine delivery to smokers that is similar to CC. In such context, NEQ levels were measured to evaluate nicotine exposure to verify if the smokers, when switching from CC, are able to get the levels of nicotine they desire when using THS *ad libitum*.

Further details on the metabolism of nicotine and its major metabolites (in the literature referred to as nicotine equivalents, or NEQ) are discussed in the nicotine factsheet [Appendix A6.1.3.17; Factsheet Nicotine].

6.1.3.1.4.11 Not Related to a Specific Chemical Entity

6.1.3.1.4.11.1 Cytochrome 1A2

Cytochrome P450 (CYP) enzymes are a superfamily of heme-containing mono-oxygenases that are found in all kingdoms of life, and show extraordinary diversity in their reaction chemistry. In mammals, these proteins are found primarily in endoplasmic reticulum of hepatocytes and other cell types, where they oxidize steroids, fatty acids and xenobiotics, and are important for the toxification, detoxification and clearance of various compounds, as well as for hormone synthesis and breakdown, cholesterol synthesis and vitamin D metabolism (McDowall 2006).

In humans, cytochrome 1A2 (CYP1A2) is constitutively expressed at higher levels only in liver and represents about 4–16% of the total hepatic P450 pool (Raunio 2012, Zanger 2013). CYP1A2 mRNA content in the liver has shown up to 40-fold variability between individuals, with corresponding variation in the enzyme activity and drug metabolism (Faber 2005).

The elimination of a large number of drugs depends mainly on CYP1A2 activity (described elsewhere (American Pharmacists Association 2014, Indiana University 2009, Zanger 2013)). CYP1A2 is also important for the metabolism of endogenous substrates (e.g. melatonin, estrone and estradiol, bilirubin and uroporphyrinogen) (Gunes 2008), and for the elimination of environmental xenobiotics. Most importantly in the context discussed here, this enzyme can additionally activate carcinogens found in cigarette smoke, such as heterocyclic amines, mycotoxins, TSNAs, and aryl amines (Eaton 1995).

Heterocyclic and aromatic amines are initially N-oxidized by the action of cytochrome CYP1A2 in the liver (MacLeod 1997). Reactive N-acetoxy intermediates can react with DNA to form covalent heterocyclic amine-DNA adducts (MacLeod 1997). The formation of DNA adducts and the extent of tumor induction is dependent on the amount of aromatic or heterocyclic amines which are converted to the reactive metabolites. The amount of DNA adducts produced depends on the quantity and activity of the enzymes involved in the formation, including CYP1A2.

Induction of CYP1A2 by smoking is well established and has been shown in *in vivo* studies (Dobrinas 2011, Ghotbi 2007). Components of cigarette smoke, likely polycyclic aromatic hydrocarbons, induce CYP1A2 activity to a different extent in various smokers (Butler 1992).

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A marked racial difference in the effect of cigarette smoking on CYP1A2 activity has also been found (Lang 1994) with CYP1A2 levels in Caucasian smokers nearly twice that of non-smokers, while African–American smokers had CYP1A2 activity approximating that of African–American or Caucasian non-smokers. These results indicate genetically based differences in the response of CYP1A2 activity to xenobiotic exposure. A study to investigate the relationship between CYP1A2 activity and the activities of other polymorphic enzymes that are involved in the metabolism of heterocyclic aromatic amines and polycyclic aromatic hydrocarbons (MacLeod 1997) showed that polymorphism of CYP1A1 and GSTM1 (i.e. glutathione S-transferase Mu 1, an enzyme often involved in detoxification of chemicals, by conjugation with glutathione) can impact the activity of CPY1A2 and that CYP1A2 activity in smokers after 3 weeks of smoking cessation showed a decrease of more than 30% in CYP1A2 activity compared to baseline (MacLeod 1997). Another smoking cessation study established an apparent half-life of CYP1A2 activity decrease of 38.6 hours (27.4-54.4 hours) (Faber 2004). Changes in the individual activities were already significantly different after 2 days of abstinence.

Besides the induction of CYP1A2 through smoking, the activity of CYP1A2 exhibit polymorphic distributions in human populations, however, the role of polymorphisms observed so far is still being discussed (Cosma 1993, Crofts 1994, Faber 2005, Ghotbi 2007, Gunes 2008, MacLeod 1997, Nakajima 1994, Zhou 2010). Individuals can be classified as slow or rapid *N*-oxidizers, depending on their activity levels of CYP1A2 (Butler 1992), with the slow metabolizer phenotype (or "poor" metabolizers) identified among Australians (5%), Japanese (14%) and Chinese (5%) (Gunes 2008). Asian and African populations have generally lower CYP1A2 activity compared with Caucasians (McGraw 2012).

Different drugs act either as CYP1A2 substrates, inducers or inhibitors. They need to be dosed with care, in particular in smokers or smokers who switch smoking status, as the metabolism of these drugs might differ between smokers and non-smokers (Faber 2005, Schaffer 2009). Slow metabolizers can end up with increased *in vivo* drug concentrations and exaggeration of drug effects (Faber 2005). For example, higher dose-adjusted plasma levels of clozapine (an atypical antipsychotic medication) in women compared with men have been reported in different study populations (Gunes 2008). Changes in diet, lifestyle, concomitant drug intake or environment should also be considered during the course of a treatment. Smoking cessation, and even a change in coffee consumption, has occasionally led to adverse drug effects through elevated plasma concentrations (Faber 2005).

Probe drugs or endogenous substances (such as phenacetin, caffeine, theophylline or melatonin) have all been successfully used to test CYP1A2 activity; however, caffeine is the most commonly used (Faber 2005). Measuring the ratio of caffeine metabolites reflects well the activities of CYP1A2 (Butler 1992).

Further details on the metabolism of CYP1A2 are discussed in the CYP1A2 factsheet [Appendix A6.1.3.18; Factsheet CYP1A2].

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6.1.3.1.4.11.2 Cytochrome 2A6

The CYP2A6 protein is mainly expressed in the liver, where it represents approximately 10% of the total liver CYP content, although with marked inter-individual variability (Raunio 2012). CYP2A6 mRNA has been detected in extrahepatic tissues, such as nasal mucosa and other areas of the respiratory tract (Koskela 1999, Raunio 2012). In the nasal mucosa and other respiratory tissues, considered as the portal-of-entry for xenobiotics, CYP2A6 may contribute to short- and long-term toxicity of compounds that are metabolized by these enzymes (Ding 2003, Hukkanen 2002).

About 40% of human drug metabolism is carried out by the CYP polymorphic enzymes (Raunio 2012), which can result in highly variable drug pharmacokinetics (Rautio 2003). Subjects carrying certain variant alleles may thus suffer from adverse effects of certain drugs because of the presence of defective alleles (Rautio 2003). Because CYP2A6 is a highly polymorphic gene, the different drugs that act either as CYP2A6 substrates, inducers or inhibitors need to be dosed with care (e.g. tegafur or SM-12502, a platelet-activating factor receptor antagonist) (Rautio 2003, Wilkinson 2005, Zevin 1999). Lists of substrates, inhibitors or activators are described elsewhere (American Pharmacists Association 2014, Nunoya 1996, Raunio 2012, Zanger 2013).

In humans, about 70–80% of nicotine is converted to cotinine. This transformation involves 2 steps (Benowitz 2009, Hukkanen 2005, Raunio 2012). The first is mediated primarily by CYP2A6 to produce nicotine-Δ1′ (5′)-iminium ion, which is in equilibrium with 5′-hydroxynicotine. The second step is catalyzed by a cytoplasmic aldehyde oxidase, yielding cotinine. CYP2A6 is the sole mediator of the conversion of cotinine to the metabolite trans-3'-hydroxycotinine (3HC) (Dempsey 2004 , Nakajima 1996). Of the abbreviated list of HPHCs (FDA (Food and Drug Administration) 2012), besides nicotine, 1,3-butadiene, NNK and NNN are also metabolized by CYP2A6 (and other enzymes) (Gregg 2013, Rossini 2008, Yamazaki 1992). Levels of their metabolites in smokers may thus vary depending on the gene alleles carried by a subject.

The availability of a phenotypic marker of CYP2A6 activity is important because of the wide variability in nicotine clearance among people with wild-type CYP2A6 genes (Benowitz 2009), and only a small proportion of the genetic variability in nicotine clearance can be explained by known CYP2A6 gene variants, at least in Caucasians (Swan 2005). The 3′-hydroxycotinine/cotinine (3HC/COT) molar metabolic ratio can be used to phenotype nicotine metabolism and CYP2A6 enzyme activity in smokers while smoking their usual cigarettes, and has even been studied as predictor of response to non-nicotine cessation pharmacotherapy such as bupropion (Benowitz 2009, Ray 2009). This 3HC/COT molar metabolic ratio (also known as the nicotine metabolite ratio, NMR) measured in human plasma, is thus a reliable measure of *in vivo* CYP2A6 activity among regular smokers (Tanner 2015). This ratio can be expressed in decimal or percentage. Because of the high genetic polymorphism of CYP2A6, NMR can serve as a "genetically-informed" biomarker of CYP2A6 activity, incorporating environmental influences on nicotine clearance (Bough 2013). NMR has recently been used to characterize novel CYP2A6 alleles (Mwenifumbo 2008).

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NMR measurements have shown that some smokers have slower nicotine metabolism (NMR < 0.31) than others (NMR ≥ 0.31) (Chenoweth 2015), with slow metabolizers being less likely to become regular smokers and, as smokers, they are less dependent and smoke fewer cigarettes per day (Chenoweth 2014, Schoedel 2004, Tyndale 2002, Tyndale 2001, Wassenaar 2011). NMR is associated with a variety of smoking behaviors, including cessation (Lerman 2006, Patterson 2008, Schnoll 2009) and early symptoms of withdrawal and craving (Lerman 2006). Lower NMR is associated with lower dependence scores (Schnoll 2014) and nicotine-mediated reward (Sofuoglu 2012), as well as with higher quit rates upon placebo and nicotine patch therapy (Lerman 2006, Schnoll 2009).

Smoking prevalence, cigarette consumption and the risk of developing smoking-related diseases differ among racial groups (Dempsey 2013). It has been shown, for example, that Asian-Americans are less likely to become smokers, smoke fewer cigarettes per day and are less likely to develop lung cancer compared to Caucasians (Benowitz 2002, Haiman 2006). Black or African Americans have a similar prevalence of smoking, smoke fewer cigarettes per day but are more likely to develop lung cancer compared to Caucasians (Haiman 2006, Trinidad 2009). One factor influencing individual differences in smoking behavior is likely to be the rate of metabolism of nicotine. Compared to Caucasians, Asians and Black or African Americans have a higher prevalence of CYP2A6 gene alleles that are associated with slower metabolism of nicotine (McDonagh 2012). In addition to the influence of ethnicity, higher metabolism of nicotine has been seen in females compared to males (Higashi 2007). Diet can also influence CYP2A6 activity (e.g. grapefruit or quercetin-containing food such as fruits or vegetables) (Chen 2009, Hukkanen 2006).

Because the elimination rate of 3HC is limited by its formation, and given that cotinine has a relatively long half-life (averaging 16 h), the NMR is expected to be stable over time among smokers who maintain constant cigarette (or tobacco) consumption (St Helen 2013). The NMR measured in various biological media was found to be stable in *ad libitum* smokers over study periods ranging from a few days to 44 weeks (St Helen 2013).

Further details on the metabolism of CYP2A6 are discussed in the CYP2A6 factsheet [Appendix A6.1.3.19; Factsheet CYP2A6].

6.1.3.1.4.11.3 Urine Mutagenicity

Urinary mutagenicity can be considered as a BoExp, as the mutagen load of urine samples indicates exposure to mutagenic compounds. It is, however, not specific of one specific mutagen, but reflects the exposure to any mutagenic compound that has been absorbed and excreted in urine. Mutagenic activity can best be tested using a procedure described in a series of papers in the early 1970s by Bruce Ames and his group, and thus often referred to as the Ames test (Ames 1975). The test procedure is well defined by the "OECD guideline for testing of chemicals: bacterial reverse mutation test (test 471)" (adopted in 1997) (OECD (Organization for Economic Co-operation and Development) 1997). Urine mutagenicity has been claimed to be highly relevant for exposure to tobacco smoke because: 1) it reflects exposure to potential mutagens, 2) it reaches beyond the

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measurement of individual chemicals to capture an integrative toxicological response in an integrative biological matrix (24-h urine), and 3) it follows a clear dose–response with cigarette consumption, even in a short time-frame (Theophilus 2015). Furthermore, the output is a combination between the exposure dose to the mutagenic agent and the metabolism of those toxicants within the exposed subject (Gregg 2013). Furthermore, it is not based on chemical standards and its characteristics as a bioassay (such as linearity, precision, accuracy, limits of detection, etc.) clearly differ from the methods used for more classical BoExp (Gregg 2013). Reference cultures, standards and frequent inter-laboratory comparisons are a requirement to ensure robust data.

Proteins and amino acids are known precursors in tobacco leaves for a number of toxicants in smoke, including aromatic amines, such as 1-NA, 2-NA, 4-ABP or *o*-toluidine (Crooks 2015) that contribute to mutagenicity. Polycyclic aromatic hydrocarbons, such as benzo[a]pyrene are also well described mutagens (IARC (International Agency for Research on Cancer) 2010d). The mutagenicity of cigarette smoke condensate can be influenced by several factors, such as tobacco type, stalk position, age, sugar and nitrogen content of tobacco leaves (DeMarini 1983). Mutagenicity may also be affected by the "draw resistance", filters and other physical characteristics (Sato 1977). Finally, the effect of pyrolysis temperature has been shown to greatly affect the mutagenicity of tobacco smoke condensate, with lower temperatures producing much less mutagens than higher temperatures (White 2001).

Since its publication, the Ames test for urinary mutagens has been adapted to be used as a non-invasive way of evaluating human exposure to mutagens, including tobacco smoke (Yamasaki 1977). The vast majority of studies to evaluate human exposure to mutagens have been conducted with urine specimens. The half-life of the smoking-related urine mutagenic activity was reported to be in the range of 7–23 h (Kado 1985) and is dependent on the half-life of the mutagens absorbed as well as metabolic inter-individual differences. In the case of smoking abstinence, the urine mutagenic activity was reported to reach non-smoker levels after 8 days (Miura 2015). Smoking abstinence studies have shown decreased urinary mutagenicity by 4- to 6-fold (Miura 2015, Roethig 2008, Smith 1996, Tricker 2012b) and up to more than 20-fold (Sarkar 2010, Theophilus 2015). These differences were not linked to the duration of the study.

Further details on the metabolism of urine mutagenicity are discussed in the urine mutagenicity factsheet [Appendix A6.1.3.20; Factsheet Urine Mutagenicity].

6.1.3.1.5 Biomarkers of Exposure in Smokers and Non-Smokers Not Assessed in PMI's Clinical Studies

This section describes the BoExp that have not been assessed in PMI's clinical studies included in this submission, or those that were used in past studies but that have been abandoned because they did not fulfill one or more of the selection criteria listed previously [Section 6.1.3.1.2], such as 1) endogenous production, 2) ubiquitous exposure, 3) lack of specificity in smokers vs non-smokers, 4) lack of robust and validated bioanalytical method, and 5) lack of representative BoExp.

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For more information, factsheets for isoprene, formaldehyde, acetaldehyde, acrylamide, and ammonia, with their main characteristics, are provided in the appendixes [A6.1.3.21 to A6.1.3.25].

6.1.3.1.5.1 Isoprene

Isoprene has been classified by IARC as "possibly carcinogenic to humans" (Group 2B) (IARC (International Agency for Research on Cancer) 1999) and by FDA as carcinogenic (U.S. Department of Health and Human Services 2012c). No human carcinogenicity data were available to the IARC Working Group (IARC (International Agency for Research on Cancer) 1999). Isoprene was tested for carcinogenicity in mice and rats in one-year inhalation studies (IARC (International Agency for Research on Cancer) 1999, National Toxicology Program (NTP) 1999). Based on animal cancer data, isoprene and chloroprene are listed in the National Toxicology Program's (NTP's) Report on Carcinogens (RoC) as reasonably anticipated to be a human carcinogen (U.S. Department of Health and Human Services 2011). Epidemiological data for isoprene are not considered adequate to evaluate the potential carcinogenicity of these agents in humans (Melnick 2001). Isoprene is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), but not of the WHO-9 list of 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

Isoprene is formed endogenously in humans and is generally the major hydrocarbon in human breath (representing up to 70% of the constituents in this matrix). The biotransformation of isoprene is similar to that of 1,3-butadiene, which involves initial oxidation to 1,2-epoxy-3-butene followed by hydrolysis to 3-butene-1,2-diol, conjugation with glutathione, or further oxidation to diepoxybutane (Gervasi 1990).

About 90% of isoprene produced endogenously in humans is metabolized and 10% is exhaled unchanged. About 75% of the total metabolites is excreted in urine, which is independent of inhaled isoprene concentration (National Toxicology Program (NTP) 1999, OECD (Organization for Economic Co-operation and Development) 2005b).

Because of the high amount of endogenous production and of its very short half-life, isoprene levels in humans have not been reported as being reliable to distinguish between smokers and non-smokers. Isoprene in exhaled breath has been shown to be slightly increased in smokers, when compared to non-smokers, however the increase was small (< 2-fold) (Euler 1996).

Therefore isoprene is not a suitable BoExp in the context of the assessment of a candidate MRTP such as THS.

6.1.3.1.5.2 Formaldehyde

Formaldehyde has been classified by IARC as carcinogenic to humans (Group 1) (IARC (International Agency for Research on Cancer) 2006) and by FDA as a carcinogen and respiratory toxicant (U.S. Department of Health and Human Services 2012c). Epidemiological studies of the relation of occupational exposure to formaldehyde and cancer at various sites provide sufficient evidence that formaldehyde causes

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nasopharyngeal cancer in humans. There is also a strong indication for a causal association between leukemia and occupational exposure to formaldehyde, and limited epidemiological evidence that formaldehyde causes sinonasal cancer in humans (U.S. Department of Health and Human Services 2010, WHO Study Group 2008). Formaldehyde has irritating effects in the upper respiratory tract and can cause nose/throat irritation leading to nasal flow resistance and laryngitis, as well as eye irritation (Toxicology Data Network 2006). It is one of the 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008), and it is also part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012).

Formaldehyde is ubiquitous in the environment (National Center for Biotechnology Information (NCBI)) and occurs as an endogenous chemical in most life forms, including humans. It is formed naturally in the troposphere during the oxidation of hydrocarbons. The highest levels of airborne formaldehyde have been detected in indoor air, where it is released from various consumer products such as building materials and home furnishings (U.S. environmental Protection Agency (EPA) 2000).

Daily endogenous turnover of formaldehyde has been estimated to be around 878-1310 mg/kg BW per day (European Food Safety Authority (EFSA) 2014). Being normal components of intermediary metabolism, neither formaldehyde nor formate are stored to any significant extent in any tissue of the body, thus storage is not a factor in its toxicity (U.S. Department of Health and Human Services 1999). The metabolism of formaldehyde to formate (via formaldehyde dehydrogenase, FDH) takes place in all tissues of the body as a consequence of its endogenous formation, and the formate is quickly removed by the supporting blood supply. Formaldehyde half-life in humans is very short and has been estimated to be within 1-1.5 min (European Food Safety Authority (EFSA) 2014).

Exposure to formaldehyde comes from a number of atmospheric environments and makes it a poor marker of exposure to tobacco smoke, due to its lack of specificity (U.S. Department of Health and Human Services 1999). Its short half-life, ubiquitous occurrence and endogenous formation, prevent a reasonable approach for formaldehyde biomonitoring which would target smoke as a specific source, and thus explain the lack of publications with data on exposure levels in smokers vs. non-smokers. Potential BoExp to formaldehyde, such as thiazolidine-carboxylic acid (Hemminki 1982), DNA-protein cross links (Shaham 1996) or DNA adducts (Wang 2009), are all affected by the large amounts of endogenous formaldehyde, as well as from the other sources of exposure to formaldehyde.
Consequently formaldehyde was not retained for assessment in PMI clinical studies for THS.

6.1.3.1.5.3 Acetaldehyde

IARC has classified acetaldehyde as “possibly carcinogenic to humans” (Group 2B) (IARC (International Agency for Research on Cancer) 1999), and FDA as a carcinogen, respiratory toxicant and addictive tobacco constituent (U.S. Department of Health and

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Human Services 2012c). An increased relative frequency of bronchial and oral cavity tumors was found in chemical workers exposed to various aldehydes (IARC (International Agency for Research on Cancer) 1999). Esophageal tumors have been associated with genetically determined, high metabolic levels of acetaldehyde after drinking alcohol (IARC (International Agency for Research on Cancer) 1999). Acetaldehyde is one of the 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008), is also part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012). Acetaldehyde vapor is irritant and causes coughing and a burning sensation in the nose, throat and eyes (IARC (International Agency for Research on Cancer) 1999). Animal studies have shown that direct administration of acetaldehyde, especially into the brain, induces several effects that mimic those of alcohol. Low doses of acetaldehyde produce behavioral effects (e.g., stimulation and reinforcement) that are characteristic of addictive drugs.

Acetaldehyde is a product of combustion and photo-oxidation of hydrocarbons commonly found in the atmosphere. It is an important industrial chemical and may be released into the air or in wastewater during its production and use. It has been detected at low levels in drinking-water, surface water, rainwater, effluents, engine exhaust and ambient and indoor air samples. Acetaldehyde is also photochemically produced in surface water. Significant exposures to acetaldehyde can occur with alcohol consumption, as it is a major metabolite of alcohol (National Center for Biotechnology Information (NCBI)). In addition, acetaldehyde is contained in plant juices, essential oils and roasted coffee (IARC (International Agency for Research on Cancer) 1999).

Acetaldehyde is metabolized to acetic acid by nicotinamide adenine dinucleotide (NAD^+)-dependent aldehyde dehydrogenase (Office of pollution prevention and toxics 1994). The rate of metabolism to acetic acid varies, but it is generally considered to be rapid. Acetic acid enters the metabolic pool of intermediary metabolism and is used in the production of carbon dioxide and water or in cellular synthesis of cholesterol, fatty acids, and other tissue constituents. Because the major metabolite of acetaldehyde enters into intermediary metabolism, the excretion of the parent compound or its metabolites is limited. Acetaldehyde has a half-life of less than 15 minutes in the circulation (Aggregated Computational Toxicology Resource (ACToR)), and its urinary excretion in rats has been shown to occur within seconds to minutes (Office of pollution prevention and toxics 1994). No accumulation in body tissues has been described (Seeman 2002).

No established BoExp to acetaldehyde from smoking exists (IARC (International Agency for Research on Cancer) 2004). In addition, acetaldehyde levels in blood were not different between smokers and non-smokers, so far as these could reliably be determined (Helander 1991, Jauhonen 1982, McLaughlin 1990).

For these reasons, acetaldehyde was not retained for assessment in PMI clinical studies for THS.

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6.1.3.1.5.4 AAMA and GAMA - Acrylamide

Acrylamide has been classified by IARC as "probably carcinogenic to humans" (Group 2A) (IARC (International Agency for Research on Cancer) 1994) and by FDA as a carcinogen (U.S. Department of Health and Human Services 2012c). It is part of the "FDA-93" established list of HPHCs in tobacco products (U.S. Department of Health and Human Services 2012c), but is not on the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), nor on the WHO-9 list of 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

Supporting evidence from human studies on cancer risk from acrylamide in food, which include case-controlled studies and reports from ongoing prospective cohort studies, found no significant associations between dietary acrylamide and the risk of cancers; however, risks for selected cancers (pancreas and Hodgkin's disease (IARC (International Agency for Research on Cancer) 1994)) were slightly elevated in a few instances (U.S. Department of Health and Human Services 2012b). In contrast, animal studies provide clear evidence of acrylamide-induced carcinogenicity. Acrylamide is formed in foods that are rich in carbohydrates (particularly potatoes) when they are fried, grilled, or baked at normal cooking temperatures. Ingestion of foods that contain acrylamide is a primary source of exposure for the general public (U.S. Department of Health and Human Services 2012b).

Acrylamide metabolism is relatively rapid. Acrylamide reacts readily with glutathione to form a glutathione conjugate, which is further metabolized to N-acetyl-S-(2-carbamoylethyl)-cysteine (AAMA) (U.S. Department of Health and Human Services 2012b, Urban 2006). Another initial step, likely catalyzed by CYP2E1, involves oxidation of acrylamide to the epoxide intermediate glycidamide (Settels 2008) which is thought to be the ultimate carcinogenic metabolite of acrylamide (Besaratinia 2005). Glycidamide can react with glutathione to form conjugates that are further metabolized to 2 isomeric mercapturic acids, namely, N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-cysteine (GAMA) and N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)-cysteine (GAMA-2). Both acrylamide and glycidamide react with nucleophilic sites in macromolecules (including hemoglobin and DNA) in Michael-type additions, producing adducts that can be measured (e.g., AAVal, GAVal) (Schettgen 2004).

Urinary excretion follows a 2-phase kinetics. Elimination half-lives of both AAMA and GAMA have been estimated to be approximately 3.5 h for the first phase and more than 10 h, up to a few days for the second phase (Boettcher 2006).

Only a few studies assessing AAMA and or GAMA metabolites in the urine of smokers and non-smokers are available. Boettcher et al. reported about 4-fold differences in AAMA and GAMA levels between smokers and non-smokers (Boettcher 2005). A recent study, in confinement, with a controlled diet, showed differences of almost 6-fold (Pluym 2015). Such high differences were, however, not found in a study by Li et al., where differences were less than 2-fold (Li 2005). In a study by Urban et al. (Urban 2006), smokers had 2.5- and 1.7-fold higher levels of AAMA and GAMA than non-smokers, respectively, while their GAMA/AAMA ratios were significantly lower compared to

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non-smokers. The GAMA/AAMA ratio is often reported, since glycidamide is thought to be the ultimate carcinogenic metabolite of acrylamide (Besaratinia 2005). In smokers, urinary excretion rates were significantly correlated with markers of smoking behavior, such as daily cigarette consumption, nicotine equivalents in urine, cotinine in saliva, and CO in exhaled breath (Urban 2006).

In two 7-day smoking abstinence studies, AAMA levels were very moderately decreased (1.3- to 2-fold) and similar decreases were found for GAMA levels (Tricker 2012a, Tricker 2012b). A 6-day study in confinement showed a decrease of about 2-fold of AAMA levels after smoking abstinence (Tricker 2012c). Furthermore, in a recent smoking abstinence study of 5 days, AAMA levels were decreased by 1.3-fold, and GAMA by 1.7-fold (Theophilus 2015). In this study, the relationship between the levels of BoExp and the number of cigarettes per day (cpd) was also studied, and both GAMA and AAMA showed a correlation with cpd, but quite weak as compared to other BoExp.

As ingestion of foods that contain acrylamide is the primary source of exposure for the general public (U.S. Department of Health and Human Services 2012b) and would considerably confound the levels observed in smokers switching from CC to THS with a likely wide variability. For this reason, acrylamide and its BoExp AAMA and GAMA were not retained for assessment in PMI clinical studies for THS, even though it had been tested in past PMI's clinical studies.

6.1.3.1.5.5 Ammonia

Ammonia is not classified as carcinogenic to humans. FDA has classified it as a respiratory toxicant (U.S. Department of Health and Human Services 2012c), as it is a known upper respiratory irritant in humans (U.S. Department of Health and Human Services 2004). It is part of the list of the FDA-18 HPHCs to report (FDA (Food and Drug Administration) 2012), but not of the WHO-9 list of 9 HPHCs suggested by the WHO Working Group on Tobacco Regulation for mandated lowering in cigarette smoke (WHO Study Group 2008).

Ammonia is naturally produced and used by all mammals in their normal metabolism. Most of the ammonia is produced by organs and tissues, but some is produced by bacteria that reside in the intestines. Ammonia is a natural compound, as well as a manufactured compound. In nature, most ammonia probably comes from decomposing animal excreta, with the decay of organic materials from plants and dead animals also contributing significant amounts. Ammonia is also exhaled by animals. For the general population, the most likely source of external exposure to elevated levels of ammonia is from the use of household cleaners containing ammonia or ammonium salts.

The human body has multiple effective mechanisms to detoxify and excrete ammonia. When absorbed through the gastrointestinal tract, it is excreted primarily in the urine as urea and other urinary nitrogen compounds, but also as urea in feces, and as a component of sweat. Ingestion of ammonium salts leads to almost complete conversion to urea in the liver, whereas exposure by other routes may lead to its metabolism in body tissues to glutamine or tissue protein (U.S. Department of Health and Human Services 2004). The estimated half-life of injected ammonia is less than 3 minutes (Brigham and Women's

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Hospital Revised 2016, Society of Nuclear Medicine and Molecular Imaging 2012). Data regarding the excretion are limited, but it is known that ammonia inhaled at low levels is exhaled primarily unchanged in the expired breath (U.S. Department of Health and Human Services 2004).

There is no known specific BoExp to ammonia in humans or animals. Furthermore, no evidence for alterations in clinical indices of body ammonia or nitrogen levels after exposure to exogenous ammonia has been reported (U.S. Department of Health and Human Services 2004). Due to the rapid clearance of ammonia from the body or its metabolization to compounds found endogenously at appreciable levels, ammonia is not a suitable HPHC to measure in the context of the assessment of a candidate MRTP such as THS.

6.1.3.1.5.6 DNA and Protein Adducts

Many xenobiotics are metabolized to reactive electrophilic intermediates, which react with nucleophilic groups of DNA and proteins as well as with other cellular nucleophiles. Adduct levels have been reported to be consistently higher in smokers than in non-smokers (reviewed in (Hecht 2003, Lodovici 2009, Wiencke 2002)). Adducts such as those formed in the DNA of white blood cells, and adducts formed with hemoglobin in red blood cells or with serum albumin can therefore be considered as another type of BoExp. Adducts seem to provide objective measures for the uptake of potential carcinogens (Besaratinia 2013, Hatsukami 2006, Hecht 2003), although not all studies indicate that DNA- and protein-adduct levels are associated with cancer incidence (Hatsukami 2006, Lodovici 2009). The most pertinent studies seem to be those where adducts are measured within cancerous tissues (Wiencke 2002), however, this is very invasive and not applicable for exposure studies. Additionally, seasonal variation as well as ambient air quality and dietary sources can induce biases in the interpretation of the adduct levels in human populations (Wiencke 2002).

Several studies have documented the existence of around 50 different types of DNA adducts in human tissues, from multiple different DNA damaging agents. Many of these DNA adducts are formed from HPHCs that are known to be carcinogenic in animal models (Poirier 2012), but are not all proven to be human carcinogens, while others are clearly associated with a particular human cancer (Jarabek 2009, Phillips 2005). Physiological cellular repair enzymes have the ability to remove DNA adducts, returning it to its normal state (Hecht 2003). However, if the adducts persist during DNA replication, miscoding can occur, thus leading to permanent mutation in the DNA sequence. Cells with damaged or mutated DNA can be removed by apoptosis, driven by the immune system, however, if the mutations occur in particular regions of crucial genes, such as the RAS or MYC oncogenes or TP53 or CDKN2A tumor-suppressor genes, the result can be loss of normal cellular growth-control regulation and tumor development (Hecht 2003).

Tobacco-specific nitrosamines have clearly emerged as one of the most important groups of carcinogens in tobacco products. Thus, their adducts appear as good indicators of their uptake and may be more specific to tobacco smoke than other HPHCs, even though

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dietary or endogenously formed nitroso compounds could also contribute to these adduct levels (Hecht 1999a).

DNA adducts are central to the carcinogenic process induced by tobacco products, but it has been acknowledged that there are still significant measurement challenges, such as accuracy and sensitivity of the methods, however more accurate and higher throughput methods should help resolve this in the near future (Hecht 2012, Hecht 2008, Hecht 2003, Kiianitsa 2014). Furthermore, adduct half-lives can be extremely long (from weeks up to 1.7 years for PAH-DNA adducts) (Hecht 2008, Wiencke 2002), making them unsuitable as BoExp in the context of relatively short-term clinical studies. Protein adducts are interesting alternatives, but their role in carcinogenic processes is not so clear, and rather serve a surrogate BoExp to potential carcinogens. Methodological limitations are such that they may not be sensitive enough to monitor low exposures (Arnold 2013).

6.1.3.1.6 Biomarkers of Exposure in Non-Clinical Studies

This section describes the approach used to characterize exposure assessment in non-clinical studies. Non-clinical *in vivo* safety studies were performed in rats according to the Organization for Economic Co-operation and Development (OECD) Testing Guideline 413 (OECD (Organization for Economic Co-operation and Development) 2009), while studies with the objective of assessing the harm reduction potential of PMI's candidate MRTPs in animal models of disease were conducted in mice. Furthermore, studies using human organotypic tissue cultures of airway epithelia, grown at the air-liquid interface, were used in systems toxicology studies to compare the impact of PMI's candidate MRTPs with that of cigarette smoke on key mechanisms of toxicity.

The rationale for the choice of BoExp measured in non-clinical *in vivo* studies is based on the same principles that apply for biomonitoring in clinical studies (see section 6.1.3.1.2). Representative metabolites of HPHCs from the particulate and gas-vapor phase of the smoke/aerosols that meet PMI's selection criteria described previously in this document have been considered. In particular, those BoExp needed to be measurable by analytical methods with a sufficient sensitivity to cover determinations in both CC smoke- and THS-exposed animals. As availability of toxicokinetic data in rodent models is limited and metabolization of reactive smoke/aerosol HPHCs is often rapid and availability of samples in sufficient quantity limited, 24h-urine was used for the analysis of (water soluble) metabolites of the following constituents:

- 3-HPMA for acrolein
- S-PMA for benzene
- CEMA for acrylonitrile
- NNAL for NNK
- Five selected nicotine metabolites including cotinine, nicotine-N'-oxide, nornicotine, norcotinine and 3'-hydroxycotinine for nicotine

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In blood, COHb as BoExp for carbon monoxide was measured as well as nicotine and cotinine as BoExp for nicotine exposure. The variability of the results for nicotine in blood was usually relatively high due to the rapid metabolization of nicotine and because of the fact that blood samples could not be drawn immediately after removal of the animals from the exposure chambers, as blood sample collection requires anesthesia, which takes several minutes to induce.

One important limitation for the determination of multiple BoExp in non-clinical studies is driven by the available volumes of body fluids in rodents, as well as the technical challenges related to the sample collection. Moreover, the sensitivity of the available analytical methods that have often been developed and validated for human samples may pose additional challenges. Given the low levels of HPHCs in THS and the short half-lives in blood for some of them (e.g. nicotine), determining BoExp of the HPHCs in urine, where more stable metabolites can be determined, was clearly also in animal studies the recommended approach. Similar to assessment in humans, the collection of urine over a 24-h period to assess the levels of exposure presents the appropriate approach, limiting the impact of the limited knowledge of the ADME of the different metabolites of interest. This collection period includes the exposure (up to 6 hours) and post-exposure phases. Rats are more suitable than mice for collection of 24-h urine, given the volume of urine produced and the availability of modified exposure tubes (for nose-only exposure) used during exposure together with suitable metabolic cages to cover post-exposure periods. Mice produce very limited urine volumes, which often dry before collection, thus posing additional challenges for quantitative assessment. Nevertheless, urine was collected in the mouse studies, but collection during exposure and after the daily exposure period was not possible. Therefore, data are provided from urine collected during the 18-h post-inhalation period only. For the determination of BoExp in blood, the same volume-driven limitations apply, with a better outcome when using rats than mice.

Furthermore, it should be considered that the main purpose of the determination of BoExp for these studies was to demonstrate proper exposure and uptake of HPHCs (including nicotine) in the animal models used, considering the specific mode of exposure by inhalation as compared to assess reduction of exposure to HPHCs as a primary objective. As the pharmacokinetic/toxicokinetic properties of BoExp to HPHCs are not known or insufficiently understood in the animal species and strains used, the determination of concentrations of BoExp were not intended to calculate exposure levels *per se* but rather to demonstrate that different particulate matter and gas-vapor phase HPHCs are taken up by the animals in quantities that are reflecting their concentrations in the test atmospheres, or reflecting their yields known from analytical aerosol characterization, although some deviations from expectation may be found.

Respiratory tract retention and uptake of chemical constituents and metabolic pathways have been reported to be variable between species and route of administration as e.g. reported for benzene (Henderson 1989, Medinsky 1989). In addition, it is important to consider that upon exposing animals to aerosols, a continuous flow of aerosol is generated that is subsequently diluted to the appropriate exposure concentration, usually targeted as a certain concentration of whole aerosol nicotine in the test atmosphere (e.g. 23 µg nicotine / L aerosol) or aerosol total particulate matter (TPM). Upon diluting the

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aerosol generated by multi-port smoking machines, redistribution of particulate phase constituents to the gas-vapor phase may occur yielding aerosols that might differ from the undiluted aerosol (Pauluhn 2008, Vanscheeuwijck 2002). The distribution of constituents in the different phases of the aerosol may affect their deposition and influence ADME and organ-specific toxicity.

Exposure to sensory irritants such as those contained in conventional cigarette smoke are known to reduce the respiratory minute volume (the product of tidal volume times the respiratory frequency) in rodents (Williams 2010), a phenomenon that is not likely to be seen upon exposure to MRTPs due to the substantially reduced levels of irritants such as formaldehyde and acrolein (Moennikes 2008). Thus, exposure of rodents to similar aerosol concentrations (i.e., normalized to nicotine concentrations in the test atmosphere) may result in a different uptake of aerosol constituents, which renders a quantitative assessment of exposure very challenging. However, because of the low levels or respiratory irritants in the THS aerosol, the overall aerosol uptake is likely to be higher than the uptake of the smoke from the CC comparator at a similar test atmosphere nicotine concentration. From a toxicological point of view, and within the model system used, this phenomenon increases the safety factor of the assessment.

In conclusion, and despite the limitations indicated, measuring urinary or blood metabolite levels of HPHCs present in the particulate and gas-vapor phase of an MRTP aerosol or cigarettes smoke, allow an appropriate biomonitoring to assess the actual exposure of rodents in inhalation studies.

The BoExp assessed in PMI's *in vivo* studies are COHb, 3-HPMA, S-PMA, NNAL, and CEMA.

6.1.3.1.6.1 COHb

Steady-state concentrations of COHb correlate well with CO-concentrations in the test atmospheres in rodent inhalation studies. In order to be able to measure the COHb in steady-state conditions, measurements were performed on blood drawn from rodents towards the end of the daily exposure period (e.g. between 4.5 and 6 hours after start of the daily exposure period when rats are exposed for 6 hrs daily) (e.g. (Moennikes 2008, Phillips 2015c, Roemer 2014, Vanscheeuwijck 2002)). COHb-levels can reach up to 50 % in rodents when exposed to high concentrations of CO. In order to avoid reaching lethal COHb levels in rodent inhalation models, exposure regimens were used in which the daily exposure to cigarette smoke is interrupted by fresh air exposure periods (Phillips 2015c).

6.1.3.1.6.2 3-HPMA

Due to its chemical reactivity, acrolein is readily absorbed in the nasal epithelia of the obligate nose-breathing rodents (Cassee 1996, Schroeter 2008, Struve 2008). Since acrolein is a potent upper respiratory tract irritant, it may, however, affect the breathing frequency of rodents, and thus may affect overall aerosol uptake (Moennikes 2008, Terpstra 2003). 3-HPMA levels in urine of rodents exposed to cigarette smoke correlate well with the concentrations of acrolein in the test atmospheres (Piade 2014).

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6.1.3.1.6.3 S-PMA

Retention and saturating concentrations of benzene when applied via injection or the inhalation route are different in mice and rats, but a linear relationship between the benzene concentration in test atmospheres and the main metabolites has been shown (Henderson 1989). Rat inhalation studies showed that steady state benzene concentrations were reached within 4 hours in blood, 6 hours in fat tissue, and less than 2 hours in bone marrow (Rickert 1979). The metabolism of benzene seems to be qualitatively similar in humans and laboratory animals (U.S. Department of Health and Human Services 2007b).

6.1.3.1.6.4 NNAL

Excretion studies of NNK metabolites in rodents (Morse 1990, Richter 1994) and studies in which NNK was administered to mice in co-exposure with cigarette smoke have shown that NNAL is a major urinary metabolite of NNK in rodents (Brown 2001), similar to humans (Goniewicz 2011, Meger 1996). When nicotine and cotinine are co-administered with NNK in rodents, NNAL formation is increased (Richter 2002). Elimination kinetics of NNK metabolites in rodents is rapid (Morse 1990) and almost quantitative in 24 h. NNAL is analyzed in rodent urine based on the method described by Kavvadias et al. (Kavvadias 2009).

The low concentrations of NNAL in rodent urine upon smoke/aerosol exposure may pose quantification issues, especially when the rodents have been exposed to aerosols from candidate MRTPs containing very low levels of TSNAs.

6.1.3.1.6.5 CEMA

Although acrylonitrile was not measured in PMI's inhalation studies with rodents, there is a linear relationship between the calculated test atmosphere concentrations of acrylonitrile based on acrylonitrile to nicotine levels from analytical smoking data and the quantities of CEMA measured in 24h-urine.

6.1.3.1.6.6 Nicotine Metabolites

For inhalation studies in which the toxicity is compared between different CCs, TPM has historically been used as a dosing parameter (e.g. (Coggins 2011, Vanscheeuwijck 2002)). In studies in which the hazard caused by MRTPs is compared to that of reference cigarettes, exposing laboratory animals to equal concentrations of TPM in the test atmospheres may not be the most appropriate approach, as the composition of the TPM is dramatically different. Other bases for comparison have thus been suggested (Moennikes 2008, WHO Study Group 2008); for nicotine-containing products, PMI has chosen to expose laboratory animals using nicotine test atmosphere concentrations as the dosing parameter (e.g. (Kogel 2014b, Phillips 2015b)).

In order to characterize the uptake of aerosols in comparative CC-MRTP animal studies, nicotine metabolites were determined in 24h-urine to cover a full daily exposure duration (typically 4-6 hours) and excretion, as well as blood nicotine and cotinine levels in the last third of the daily exposure period (given the short half-life of nicotine in blood, measurements are done in steady-state condition).

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The 5 most abundant urinary nicotine metabolites, as determined after *i.v.* injection of nicotine in rats (Schepers 1993) were analyzed in rodent urine after derivatization with 1,3-diethyl-2-thiobarbituric acid (Rustemeier 1993), and represented approximatively 50 % of the total amount administered (Schepers 1993): cotinine, nicotine-N'-oxide, nornicotine, norcotinine and 3'-hydroxycotinine. The total amount of nicotine metabolites in the urine correlates well with the amount of nicotine in the test atmospheres (e.g. (Kogel 2014a, Phillips 2015a, Terpstra 2003, Werley 2008)).

Nicotine and cotinine concentrations were also determined in rodent blood. Due to the short half-life of nicotine in rat blood (independent of the mode of exposure, 20 – 60 min, (Abobo 2012, Craig 2014, Kyerematen 1988, Sastry 1995)), correlation with nicotine test atmosphere concentrations is rendered challenging because of the duration of blood sample collection procedures (including anesthesia) (Werley 2014), whereas cotinine concentrations in blood are more suitable for biomonitoring because of its longer half-life (300 min or longer, (Kyerematen 1988, Sastry 1995)). For studies in mice, the short half-life of nicotine (6-30 min, (Alsharari 2014, Klein 2004)) causes even more technical problems, whereas using blood cotinine as a biomonitoring parameter is more suitable (half-life over 100 min (Alsharari 2014)).

Section 6.1.3.1.4.10.1 provides overall information on nicotine and its metabolism. COHb concentrations are easily measured using spectrophotometric measurements of absorbance at several wavelengths of mixed hemoglobins in hemolysate. The accuracy of the measurement is lower at low hemoglobin proportions (overestimated at COHb proportions of COHb <2.5 % (Mahoney 1993)).

6.1.3.1.6.7 Menthol

In PMI's inhalation studies, using diluted mainstream smoke or aerosol from candidate MRTPs, menthol was found to be predominantly (approximately 90%) in the gas-vapor phase (unpublished results). The principal menthol metabolite identified in rat plasma and urine following oral menthol administration is menthol glucuronide (Yamaguchi 1994). The other metabolites include mono- or di-hydroxylated menthol derivatives and carboxylic acids (predominantly p-menthane-3,8-diol and 3,8-oxy-p-menthane-7-caboxylic acid), which are excreted partly as glucuronic acid conjugates (Madyastha 1988). In human liver microsomes, cytochrome P450 (CYP) 2A6 was determined to be the major enzyme involved in the hydroxylation of menthol (Miyazawa 2011). Menthol and menthol glucuronide was measured in rat plasma and urine for up to 6 h after oral administration (Spichiger 2004), with a half-life that is probably approximating that in human, reported to be 75 min (Gelal 1999).

Menthol and p-menthane-3,8-diol were measured in plasma from menthol cigarette- and mentholated MRTP aerosol-exposed rats as a biomonitoring parameter for the exposure to menthol. Sample collection was performed during the last hour of a 6 hour exposure period.

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6.1.3.1.6.8 Not Related to a Specific Chemical Entity

6.1.3.1.6.8.1 Cytochrome 1A2

CYP1A2 was selected as a biomarker of exposure in humans (see section 6.1.3.2.4.11.1). Like in humans, CYP1A2 is also mainly expressed in the liver of mice, and regulated in similar manners. It is also inducible in mouse liver by PAHs (Ma 2007, Shimada 2006) and cigarettes smoke. We have therefore chosen to measure CYP1A2 expression in the livers (Lo Sasso 2016) of the mice involved in the Apoe$^{-/-}$ inhalation study described in [Section 6.1.4.1.1 – Clinical Risk Endpoints] (Phillips 2016). This provides us with a translational biomarker of exposure that is not linked to a specific chemical entity.

6.1.3.1.6.8.2 Cytochrome 1A1 and 1B1

In addition to CYP1A2, CYP1A1 and CYP1B1 are also induced by PAHs and are known to activate B[a]P and other PAHs to their diol-epoxides, which are highly mutagenic and carcinogenic metabolites that initiate cell transformation (Shimada 2006). These 2 enzymes have previously been shown to be strongly induced by cigarette smoke exposure in human oral mucosa biopsies (Boyle 2010) and human organotypic tissue cultures of the oral mucosa (Schlage 2014). Expression of their mRNA as well as their combined enzyme activity, therefore serve as suitable biomarkers of cigarette smoke exposure in systems toxicology-based *in vitro* studies conducted in organotypic tissue cultures of airway epithelia grown at the air-liquid interface and exposed with whole cigarette smoke of THS aerosol.

6.1.3.1.7 Published Data on BoExp Levels in Smoker, Non-Smokers, and Quitters

In this section, representative published values of the different HPHCs and related BoExp assessed in PMI's studies are provided. Values are presented as published, and were converted, when applicable, into the values used in PMI's studies (in [square brackets]). Table 5 provides data obtained in smokers and non-smokers, and Table 6 provides data obtained in smoking cessation/abstinence studies. The data presented were not meant to provide a systematic review of all published data on those HPHCs, but as a representative sample of published data.

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Table 5 Representative Levels of BoExp in Smokers and Non-Smokers – Literature Review of Cross Sectional Studies

Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Cole (Cole 1975)	COHb	1976	5.5 (2.2-13)	0.97 (0.2-2.5)	%; mean (range)
Szadkowski (Szadkowski 1976)	COHb	1979	3.37 ± 1.76	0.72 ± 0.25	%; mean ± SD
Frederiksen (Frederiksen 1979)	COHb	1982	up to 15%	0.5-2	%; range
Saloojee (Saloojee 1982)	COHb	1984	7.09 ± 2.9	0.73 ± 0.41	%; mean ± SD
Heinemann (Heinemann 1984)	COHb	1984	6.9 (4-10.2)	2.2 (1.7-2.7)	%; P_{10}-P_{90}
Jarvis (Jarvis 1984)	COHb	1984	3.94	0.9	%
Pojer (Pojer 1984)	COHb	2006	4.36 ± 2.09	0.93 ± 0.52	%; mean ± SD
Scherer (Scherer 2006a)	COHb	2009	4.0–8.0	0.8–1.5	%; range
Lowe (Lowe 2009)	COHb	2009	5.69 ± 1.82	0.35 ± 0.38	%; mean ± SD
Roethig (Roethig 2009)	COHb	2011	5.26 ± 0.04	1.45 ± 0.02	%; weighed mean ± SE
Lindner (Lindner 2011)	COHb	2015	3.63 ± 2.21	0.23 ± 0.4	%; mean ± SD
Bergen (Bergen 2015)	S-PMA	2011	5.26 ± 2.3	1.46 ± 0.5	%; mean ± SD
Lindner (Lindner 2011)	S-PMA	2005	3.27 ± 3.02 [3270 ± 3020]	0.30 ± 0.42 [300 ± 420]	µg/24h [ng/24h]; mean ± SD
Sarkar (Sarkar 2005)	S-PMA	2009	3.02 ± 2.17 [3020 ± 2170]	0.16 ± 0.20 [160 ± 200]	µg/g creat [pg/mg creat]; mean ± SD
Roethig (Roethig 2009)	S-PMA	2009	3.61 (0.1) [3610 (100)]	0.30 (0.02) [300 (20)]	µg/24h [ng/24h]; weighed mean (SE)
Scherer (Scherer 2006b)	S-PMA	2006	75.0 ± 50.0 [75000 ± 50000]	NM	µg/24h [ng/24h]
Ding (Ding 2009)	S-PMA	2009	ND-18.4 [18400]	ND-0.45 [450]	µg/g creat [pg/mg creat] (range)

(table continues)

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Shepperd (Shepperd 2013)	S-PMA	2013	4501 ± 3614 (376–18739)	117 ± 78 (40–447)	ng/24h; LS means ± SD (ranges)
Jain (Jain 2015)	S-PMA	2015	25.1 (20.0-31.4)	11.5 (10.0-13.4)	ng/mL; adjusted geometric mean (95% CI)
Eckert (Eckert 2011)	S-PMA	2011	<5.0-12.0 (<5.0-11.9) [<5000-12000 (<5000-11900)]	<5.0 (<5.0) [<5000 (<5000)]	µg/24h [ng/24h]; median (range)
Bergen (Bergen 2015)	S-PMA	2015	3.52 ± 3.3 [3520 ± 3300]	0.49 ± 1.0 [490 ± 1000]	µg/24h [ng/24h]; mean ± SD
Lindner (Lindner 2011)	3-HPMA	2011	1.81 ± 1.26 [1810 ± 1260]	0.63 ± 0.80 [630 ± 800]	mg/24h [µg/24h]; mean ± SD
Carmella (Carmella 2007)	3-HPMA	2007	3950 ± 2950 [874 ± 653]	1900 ± 3000 [420 ± 664]	pmol/mg creat [ng/mg creat]; mean ± SD
Ding (Ding 2009)	3-HPMA	2009	75-3678	ND-245	µg/g creat or ng/mg creat; mean ± SD
Shepperd (Shepperd 2013)	3-HPMA	2013	1342 ± 743	251 ± 125	µg/24h; mean ± SD
Yan (Yan 2010)	3-HPMA	2010	2442.0 ± 993.6	358.9 ± 143.1	µg/24h; mean ± SD
Yan (Yan 2010)	3-HPMA	2010	2069.7 ± 925.5	310.0 ± 167.2	ng/mg creat; mean ± SD
Eckert (Eckert 2011)	3-HPMA	2011	884 (104-4423)	146 (39.3-1562)	µg/g creat or ng/mg creat; median (range)
Schettgen (Schettgen 2008b)	3-HPMA	2008	1630 (213–4242)	112.8 (57–1090)	µg/g creat [ng/mg creat]; median (range)
Mascher (Mascher 2001)	3-HPMA	2001	2809 ± 385	812 ± 123	µg/24h; mean ± SD
Minet (Minet 2011)	3-HPMA	2011	810.7 ± 559.7 (680.4) ISO tar yield 1mg	228.4 ± 70.4 (231.6)	µg/24h; mean ± SD
Minet (Minet 2011)	3-HPMA	2011	1231.0 ± 687.0 (1129.0) ISO tar yield 4mg	228.4 ± 70.4 (231.6)	µg/24h; mean ± SD
Minet (Minet 2011)	3-HPMA	2011	1945.0 ± 968.0 (1129.0) ISO tar yield 10mg	228.4 ± 70.4 (231.6)	µg/24h; mean ± SD

(table continues)

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Morin (Morin 2011)	3-HPMA	2011	2494 (2252-2735) 14 cig/day	983 (879-1088)	µg/24h; mean (95%CI)
Ding (Ding 2009)	3-HPMA	2009	75-3678	ND-245	µg/g creat [ng/mg creat]; (range)
Scherer (Scherer 2007a)	3-HPMA	2007	1297 ± 1164	337 ± 383	µg/24h; mean ± SD
Roethig (Roethig 2009)	3-HPMA	2009	2,030 (24)	458 (17)	µg/24h; weighted mean ± SE)
Alwis (Alwis 2015)	3-HPMA	2015	1089 (469, 2012)	219 (140, 353)	µg/g creat [ng/mg creat]; sample-weighted median (25^{th}, 75^{th} percentile)
Bergen (Bergen 2015)	3-HPMA	2015	1941.9 ± 1326	416.7 ± 532	µg/24h; mean ± SD
Pluym (Pluym 2015)	3-HPMA	2015	372 (157–606)	62.5 (39.1-284)	µg/g creat; median (min-max), >10 cig/d
Jain (Jain 2015)	3-HPMA	2015	529.7 (455.5-615.9)	399.4 (351.6-453.6)	ng/mL; adjusted geometric mean (95% CI)
Lindner (Lindner 2011)	MHBMA	2011	3.27 ± 3.02 [3270 ± 3020]	0.30 ± 0.42 [300 ± 420]	µg/24h [ng/24h]; mean ± SD
Sarkar (Sarkar 2005)	MHBMA	2005	3.02 ± 2.17 [3020 ± 2170]	0.16 ± 0.20 [160 ± 200]	µg/g creat [pg/mg creat]; mean ± SD
Schettgen (Schettgen 2009)	MHBMA	2009	< 2 (8.6)	< 2 (<2)	µg/L; median (95^{th} percentile)
Roethig (Roethig 2009)	MHBMA	2009	3.61 (0.1) [3610 (100)]	0.30 (0.02) [300 (20)]	µg/24h [ng/24h]; weighed mean (SE)
Scherer (Scherer 2006b)	MHBMA	2006	75.0 ± 50.0 [75000 ± 50000]	NM	µg/24h [ng/24h]
Ding (Ding 2009)	MHBMA	2009	ND-59.7 [59700]	ND-122 [122000]	µg/g creat [pg/mg creat] (range)
Shepperd (Shepperd 2013)	MHBMA	2013	4501 ± 3614 (376–18739)	117 ± 78 (40–447)	ng/24h; LS means ± SD (ranges)
Jain (Jain 2015)	MHBMA	2015	25.1 (20.0-31.4)	11.5 (10.0-13.4)	ng/mL; adjusted geometric mean (95% CI)

(table continues)

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Eckert (Eckert 2011)	MHBMA	2011	<5.0-12.0 (<5.0-11.9) [<5000-12000 (<5000-11900)]	<5.0 (<5.0) [<5000 (<5000)]	µg/24h [ng/24h]; median (range)
Bergen (Bergen 2015)	MHBMA	2015	3.52 ± 3.3 [3520 ± 3300]	0.49 ± 1.0 [490 ± 1000]	µg/24h [ng/24h]; mean ± SD
Scherer (Scherer 2007a)	Total NNAL	2007	1538 ± 1715 [321.81 ± 358.85]	NM	pmol/24h [ng/24h]; mean ± SD
Lindner (Lindner 2011)	Total NNAL	2011	189.5 ± 161.1	3.9 ± 9.4	ng/24h; mean ± SD
Mendes (Mendes 2008)	Total NNAL	2008	582.4 ± 270.0	NM	ng/24h; mean ± SD
Shepperd (Shepperd 2013)	Total NNAL	2013	287 ± 164 (13-678) [60.05 ± 34.32 (2.72-141.86)]	22 ± 61 (1-411) [4.60 ± 12.76 (0.21-86)]	pmol/24h [ng/24h]; LS means ± SD (ranges)
Kavvadias (Kavvadias 2009)	Total NNAL	2009	204.20 ± 146.90	16.06 ± 11.99	ng/24h; mean ± SD
Lowe (Lowe 2009)	Total NNAL	2009	0.26 ± 0.10 [260 ± 100]	0.01 ± 0.01 [10 ± 10]	ng/mg creat [pg/mg creat]; mean ± SD
Lowe (Lowe 2009)	Total NNAL	2009	199.50 ± 153.90 (≤10 cpd) 362.10 ± 150.60 (≥20 cpd)	8.23 ± 6.52	ng/24h, mean ± SD
Melikian (Melikian 2007)	Total NNAL	2007	1.68 (1.41-2.0) [351.52 (295.03-418.48)]	NM	pmol/mg creat [pg/mg creat]; geometric mean (95% CI)
Mendes (Mendes 2008)	Total NNAL	2008	595.0 ± 279.9	NM	ng/24 h; mean ± SD
Sarkar (Sarkar 2008)	Total NNAL	2008	320.68 ± 185.62	NM	ng/g creat [pg/mg creat]; mean ± SD
Strasser (Strasser 2011)	Total NNAL	2011	1.47 ± 0.79 [307.58 ± 165.30]	NM	pmol/mg creat [pg/mg creat];mean ± SD
Chung (Chung 2013)	Total NNAL	2013	1.40 ± 0.71 [1400 ± 710]	0.40 ± 0.07 [400 ± 70]	µg/g creat [pg/mg creat]; mean ± SD
Hecht (Hecht 1999b)	Total NNAL	1999	0.601 ± 0.366	0.016 ± 0.029	pmol/ml; mean + SD
Sarkar (Sarkar 2008)	Total NNAL	2008	320.68 ± 185.62	NM	ng/g creat [pg/mg creat]; mean ± SD

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Lee (Lee 2008)	Total NNAL	2008	3.16 ± 0.99 [3160 ± 990]	1.49 ± 0.28 [1490 ± 280]	µg/g creat [pg/mg creat]; mean ± SE
Calapai (Calapai 2009)	Total NNAL	2009	89.62 (61.03-131-59)	6.13 (4.78-7.87)	ng/g creat [pg/mg creat]; mean (95% CI)
Calapai (Calapai 2009)	Total NNAL	2009	191.69 (143.65-255.79)	6.13 (4.78-7.87)	ng/g creat [pg/mg creat]; mean (95% CI)
Morin (Morin 2011)	Total NNAL	2011	213 (178-248)	10 (9-11) [o]	ng/24h; mean (95% CI)
Morin (Morin 2011)	Total NNAL	2011	176 (147-204)	10 (9-11) [o]	ng/24h; mean (95% CI)
Morin (Morin 2011)	Total NNAL	2011	252 (220-284)	10 (9-11) [o]	ng/24h; mean (95% CI)
Hou (Hou 2012b)	Total NNAL	2012	12.25 (1.10-58.10)	3.19 (0-17.29)	µg/mol creat; mean (range)
Hou (Hou 2012b)	Total NNAL	2012	12.74 (1.28-60.22)	3.19 (0-17.29)	µg/mol creat; mean (range)
Hou (Hou 2012b)	Total NNAL	2012	11.83 (0.97-49.13)	3.19 (0-17.29)	µg/mol creat; mean (range)
Radwan (Radwan 2013)	Total NNAL	2013	0.96 ± 0.07 [i]	0.05 ± 0.21	pmol/ml; geometric mean ± SD
Radwan (Radwan 2013)	Total NNAL	2013	1.35 ± 0.51 [j]	0.05 ± 0.21	pmol/ml; geometric mean ± SD
Radwan (Radwan 2013)	Total NNAL	2013	1.00 ± 0.36 [k]	0.05 ± 0.21	pmol/ml; geometric mean ± SD
Zhu (Zhu 2013)	Total NNAL	2013	1.1 ± 0.8 [230.16 ± 167.39]	NM	pmol/mg creat [pg/mg creat]; mean ± SD
Hu (Hu 2014)	Total NNAL	2014	0.17 ± 0.14 (0.005–0.86) [170 ± 140 (5-860)]	NM	ng/mg creat [pg/mg creat]; mean ± SD (range)
Al Ali (Al Ali 2015)	Total NNAL	2015	46.8 (27.6-79.3)	3.9 (1.6-7.9)	pg/mL ; mean (range)
Miura (Miura 2015)	Total NNAL	2015	187 ± 100	3.40 ± 3.62	ng/24h; mean ± SD
Shepperd (Shepperd 2013)	Total NNN	2013	13.9 ± 11.4 (0.4-55.7)	0.9 ± 0.9 (0.3-5.3)	ng/24h; LS Means ± SD (ranges)
Kavvadias (Kavvadias 2009)	Total NNN	2009	24.50 ± 35.83	0.49 ± 0.53	ng/24h; mean ± SD

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Stepanov (Stepanov 2005)	Total NNN	2005	0.086 ± 0.12 [15.24 ± 21.26]	NM	pmol/mg creat [pg/mg creat]; mean ± SD
Yuan (Yuan 2011)	Total NNN	2011	43.3 (33.7–55.6) [7.67 (5.97-9.85)]	NM	fmol/mg creat [pg/mg creat]; geometric mean (95% CI),
Zhu (Zhu 2013)	Total NNN	2013	0.182 ± 0.390 [32.25 ± 69.11]	NM	pmol/mg creat [pg/mg creat]; mean ± SD
Scherer (Scherer 2007a)	Total 1-OHP	2007	0.196 ± 0.145 [196 ± 145]	0.101 ± 0.070 [101 ± 70]	µg/24h [ng/24h]; mean ± SD
Shepperd (Shepperd 2013)	Total 1-OHP	2013	254 ± 101 (71-511)	105 ± 65 (37-336)	ng/24h; LS means ± SD (ranges)
Barbeau (Barbeau 2011)	Total 1-OHP	2011	162 ± 90 [313 ± 174]	42 ± 33 [81 ± 64]	nmol/mol creat [pg/mg creat]; mean ± SD
Lindner (Lindner 2011)	Total 1-OHP	2011	290.6 ± 352.2	122.5 ± 100.5	ng/24h; mean ± SD
Mendes (Mendes 2008)	Total 1-OHP	2008	232.1 ± 108.9	NM	ng/24h; mean ± SD
St. Helen (St Helen 2012)	Total 1-OHP	2012	2.76 (2.46, 3.09) [602.37 (536.90, 674.39)]	1.12 (0.96, 1.30) [244.44 (209.52, 283.73)]	pmol/mg creat [pg/mg creat]; geometric mean (95% CI)
St. Helen (St Helen 2012)	Total 1-OHP	2012	1.14 (1.03, 1.26) [248.81 (224.80, 275)]	0.51 (0.43, 0.61) [111.31 (93.85, 133.13)]	pmol/mg creat [pg/mg creat]; geometric mean (95% CI)
Buratti (Buratti 2007)	Total 1-OHP	2007	0.41 ± 0.30 (0.33) [410 ± 300 (330)]	0.28 ± 0.29 (0.18) [280 ± 290 (180)]	µg/L [pg/mL]; mean ± SD (median)
Melikian (Melikian 2007)	Total 1-OHP	2007	Low tar 378 (318-448)	NM	pg/mg creat; geometric mean (95% CI)
Melikian (Melikian 2007)	Total 1-OHP	2007	Medium tar 396 (334-470)	NM	pg/mg creat; geometric mean (95% CI)
Melikian (Melikian 2007)	Total 1-OHP	2007	High tar 347 (279-432)	NM	pg/mg creat; geometric mean (95% CI)
Jongeneelen (Jongeneelen 1988)	Total 1-OHP	1988	0.28 (0.09-1.31) [540 (174-2528)]	0.26 (0.02-0.66) [502 (39-1273)]	µmol/mol creat [pg/mg creat]; median (90% CI)

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Roethig (Roethig 2009)	Total 1-OHP	2009	317 ± 6.8	110 ± 7.1	ng/24h; weighed mean ± SE
Roggi (Roggi 1997)	Total 1-OHP	1997	0.408 ± 0.312 [408 ± 312]	0.233 ± 0.41 [233 ± 410]	µg/g creat [pg/mg creat]; mean ± SD
Sithisarankul (Sithisarankul 1997)	Total 1-OHP	1997	1.04 ± 0.13 [227 ± 28]	0.55 ± 0.05 [120 ± 11]	pmol/mL [pg/mL]; mean ± SE
Merlo (Merlo 1998)	Total 1-OHP	1998	0.176 ± 0.172 [340 ± 332]	0.089 ± 0.095 [172 ± 183]	µmol/mol creat [pg/mg creat]; mean ± SD
Scherer (Scherer 2000)	Total 1-OHP	2000	0.346 ± 0.048 [346 ± 48]	0.171 ± 0.020 [171 ± 20]	µg/24h [ng/24h]; mean ± SE
Pastorelli (Pastorelli 1999)	Total 1-OHP	1999	0.54 ± 0.31 [540 ± 310]	0.24 ± 0.24 [240 ± 240]	ng/mL [pg/mL]; mean ± SD
Jacob (Jacob 1999)	Total 1-OHP	1999	603 ± 483	218 ± 159	ng/24h; mean ± SD
Li (Li 2000)	Total 1-OHP	2000	0.46 ± 0.36 [888 ± 695]	0.04 ± 0.03 [77 ± 58]	µmol/mol creat [pg/mg creat]; mean ± SD
Nerurkar (Nerurkar 2000)	Total 1-OHP	2000	0.51 (0.34–0.69)	0.27 (0.22–0.32)	nmol/12h; geometric mean (95% CI)
Dor (Dor 2000)	Total 1-OHP	2000	0.04 (0.02-0.06) [77 (39-116)]	0.02 (0.01-0.04) [39 (19-77)]	µmol/mol creat [pg/mg creat]; median (95% CI)
Alexandrie (Alexandrie 2000)	Total 1-OHP	2000	0.17 (0.09–0.99) [328 (174-1910)]	0.10 (0.02–0.50) [193 (39-965)]	µmol/mol creat [pg/mg creat]; median (range)
Nan (Elmstahl 1991)	Total 1-OHP	1991	0.05 ± 2.04 [96 ± 3936]	0.04 ± 2.43 [77 ± 4688]	µmol/mol creat [pg/mg creat]; geometric mean ± SD
van Delft (van Delft 2001)	Total 1-OHP	2001	0.70 ± 0.39 [1351 ± 752]	0.27 ± 0.22 [521 ± 424]	µmol/mol creat [pg/mg creat]; mean ± SD
Kim (Kim 2001)	Total 1-OHP	2001	0.05 ± 0.04 [96 ± 77]	0.03 ± 0.02 [58 ± 39]	µmol/mol creat [pg/mg creat]; mean ± SD
Szaniszlo (Szaniszlo 2001)	Total 1-OHP	2001	0.57 ± 0.38 [1100 ± 733]	0.11 ± 0.05 [212 ± 96]	µmol/mol creat [pg/mg creat]; arithmetic mean ± SD
Morin (Morin 2011)	Total 1-OHP	2011	350 (315-385) [d]	91 (72-109)	ng/24h; mean (95% CI)

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Lindner (Lindner 2011)	4-ABP	2011	24.66 ± 74.44	2.77 ± 11.79	ng/24h; mean ± SD
Scherer (Scherer 2014)	4-ABP	2014	19.5 ± 8.5	2.2 ± 2.6 (2.1 ± 1.7)[a]	ng/24h; mean ± SD
Grimmer (Grimmer 2000)	4-ABP	2000	78.6 ± 85.2	68.1 ± 91.5	ng/24h; mean ± SD
Riedel (Riedel 2006)	4-ABP	2006	15.3 ± 7.3	9.6 ± 4.9	ng/24h; mean ± SD
Seyler (Seyler 2011)	4-ABP	2011	8.69 (7.43–10.16)	1.64 (1.30–2.07)	pg/mg creat; geometric mean (95% CI)
Yu (Yu 2014)	4-ABP	2014	17.27 ± 13.16	5.58 ± 2.90	ng/24h; mean ± SD
Miura (Miura 2015)	4-ABP	2015	11.0 ± 7.37	1.43 ± 1.39	ng/24h; mean ± SD
Grimmer (Grimmer 2000)	1-NA	2000	506.7 ± 670.0	68.9 ± 105.4	ng/24h; mean ± SD
Yu (Yu 2014)	1-NA	2014	67.02 ± 45.46	12.32 ± 8.91	ng/24h; mean ± SD
Riffelmann (Riffelmann 1995)	2-NA	1995	3.1 ± 2.1	0.5 ± 0.7	µg/L; mean ± SD
Grimmer (Grimmer 2000)	2-NA	2000	120.8 ± 279.2	84.5 ± 102.7	ng/24h; mean ± SD
Riedel (Riedel 2006)	2-NA	2006	20.8 ± 11.2	10.7 ± 9.5	ng/24h; mean ± SD
Lindner (Lindner 2011)	2-NA	2011	17.54 ± 50.88	1.79 ± 3.59	ng/24h; SD
Yu (Yu 2014)	2-NA	2014	47.40 ± 50.68	10.18 ± 7.25	ng/24h; mean ± SD
Shepperd (Shepperd 2013)	2-NA	2013	17.5 ± 7.6	1.6 ± 0.87	ng/24h; LS mean ± SD
Frost-Pineda (Frost-Pineda 2008)	2-NA	2008	17.9 ± 14.8	16.1 ± 8.5	ng/24h; mean ± SD
El-Bayoumi (el-Bayoumy 1986)	*o*-toluidine	1986	6357 ± 4041	4222 ± 3798	ng/24h; mean ± SD
Riffelman (Riffelmann 1995)	*o*-toluidine	1995	1.7 ± 1.6	0.0 ± 0.0	µg/L; mean ± SD

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Ward (Ward 1996)	*o*-toluidine	1996	0.9 ± 0.7	1.3 ± 1.3	µg/L; mean ± SD
Weiss (Weiss 2002)	*o*-toluidine	2001	NM	0.12 (0.05–3.1)	µg/L; median (range)
Weiss (Weiss 2005)	*o*-toluidine	2005	206 (ND-838)	85 (ND-1660)	ng/L; median (range)
Riedel (Riedel 2006)	*o*-toluidine	2006	204.2 ± 59.1	105.2 ± 25.6[a]	ng/24h; mean ± SD
Kutting (Kutting 2009)	*o*-toluidine	2009	130 (ND-173)	100 (ND-34)	ng/L; mean (range)
Lindner (Lindner 2011)	*o*-toluidine	2011	179.2 ± 491.4	63.5 ± 128.3	ng/24h; mean ± SD
Shepperd (Shepperd 2013)	*o*-toluidine	2013	149 ± 62.3 (40-277)	53.9 ± 29.6 (19.3–137.2)	ng/24h; LS Means ± SD (ranges)
Schettgen (Schettgen 2009)	CEMA	2009	240 (2-1382)	2 (<1-21.3)	ng/mL; median (range)
Schettgen (Schettgen 2012)	CEMA	2012	148 (393)	1.5 (5.9)	µg/g creat or ng/mg creat; median creat [(95 percentile)
Minet (Minet 2011)	CEMA	2011	96.9 ± 81.8 (75.4) ISO tar yield 1 mg	1.3 ± 0.7 (1.1)	µg/24h; mean ± SD (median)
Minet (Minet 2011)	CEMA	2011	139.3 ± 72.1 (140.2) ISO tar yield 4 mg	1.3 ± 0.7 (1.1)	µg/24h; mean ± SD (median)
Minet (Minet 2011)	CEMA	2011	214.8 ± 113.8 (186.6) ISO tar yield 10 mg	1.3 ± 0.7 (1.1)	µg/24h; mean ± SD (median)
Ding (Ding 2009)	CEMA	2009	27-744	ND-158	µg/g creat or ng/mg creat
Xiaotao (Xiaotao 2014)	CEMA	2014	26.34 (0.551–162)	0.641 (0 to 7.02)	ng/mL; mean (range)
Jacob (Jacob 2013)	CEMA	2013	70.9 (45.4–110.9)	NM	µg/24h; geometric mean (95% CI)
Alwis (Alwis 2012)	CEMA	2012	187 ± 181	4.60 ± 35	ng/mL; mean ± SD
Pluym (Pluym 2015)	CEMA	2015	72.5 (28.6-140.4)	0.46 (0.23-8.6)	µg/g creat or ng/mg creat; median (min-max)
Calafat (Calafat 1999)	HEMA	1999	2.8 [2800]	1.1 [1100]	µg/g creat [pg/mg creat]; geometric mean

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Schettgen (Schettgen 2008b)	HEMA	2008	4.0 (1.3-6.0) [4000 (1300-6000)]	1.7 (0.7-4.2) [1700 (700-4200)]	µg/g creat [pg/mg creat]; median (ranges)
Ding (Ding 2009)	HEMA	2009	ND-16.0 [16000]	ND-1.05 [1050]	µg/g creat [pg/mg creat]; median ranges
Eckert (Eckert 2011)	HEMA	2011	4.9 (1.1–67.7) [4900 (1100-67700)]	1.6 (0.6–8.1) [1600 (600-8100)]	µg/g creat [pg/mg creat]; median ranges
Hou (Hou 2012a)	HEMA	2012	15.5 (3.98–944) [3,212 (825-195644)]	4.4 (<LOQ–60.6)	nmol/24h; mean (ranges) [ng/24h]
Xiaotao (Xiaotao 2014)	HEMA	2014	1.18 (0.00-11.20) 8mg tar	[912 (<LOQ-12559)]	ng/ml; mean (ranges)
Xiaotao (Xiaotao 2014)	HEMA	2014	2.54 (0.00-18.8) 10mg tar	0.72 (0-10.0)	ng/ml; mean (ranges)
Xiaotao (Xiaotao 2014)	HEMA	2014	1.24 (0–7.28) 13mg tar	0.72 (0-10.0)	ng/ml; mean (ranges)
Pluym (Pluym 2015)	HEMA	2015	2.0 (1.1-4.2)	0.72 (0-10.0)	µg/g creat; median (min-max)
Alwis (Alwis 2015)	HEMA	2015	1.90 ± 3.70	1.1 (0.11-38.3)	ng/mL; mean ± SD
Lafontaine (Lafontaine 2006)	3-OH-B[a]P	2006	<0.01-0.084 (0.030; 0.023)	<0.01-0.045 (0.014; 0.011)	nmol/mol creat; arithmetic mean (median)
Zhang (Zhang 2015)	3-OH-B[a]P	2015	2.83 ± 1.78 (8mg tar) [2830 ± 1780] [a] 6.00 ± 4.44 (13mg tar) [6000 ± 4440]	1.30 ± 0.20 [1300 ± 200] [a]	ng/g creat [fg/mg creat]; mean ± SD
Hecht (Hecht 2014)	3-HMPMA	2014	4990 (2930–8490)	705 (456–1090)	pmol/ml; geometric mean (95% CI)
Zhang (Zhang 2014)	3-HMPMA	2014	1287.83 (100.53–6975)	191.9 (0.00–785)	ng/mL; mean (range)
Scherer (Scherer 2007b)	3-HMPMA	2007	2122 ± 1286	752 ± 457	µg/24h; mean ± SD
Pluym (Pluym 2015)	3-HMPMA	2015	121.7 (57.0-220.0)	18.9 (9.7-64.4)	µg/g creat [ng/mg creat]; median (min-max)
Miura (Miura 2015)	3-HMPMA	2015	1616 ± 793	257 ± 58.1	µg/24h; mean ± SD
Alwis (Alwis 2015)	3-HMPMA	2015	1546 ± 1643	406 ± 487	ng/mL; mean ± SD

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Author	BoExp	Publication year	Levels in smokers[1]	Levels in non-smokers [1]	Units; measure of dispersion [2]
Imbriani (Imbriani 1999)	S-BMA	1999	9.1 ± 9.3 [9100 ± 9300]	10. 2 ± 11.9 [10200 ± 11900]	µg/g creat; mean ± SD [pg/mg creat; mean ± SD]
Schettgen (Schettgen 2008a)	S-BMA	2008	11.5 (0.9–51.2)	8.2 (1.6–77.4)	µg/L; ranges
Lovreglio (Lovreglio 2010)	S-BMA	2010	10.27 ± 9.40 [10270 ± 9400]	8.58 ± 10.10 [8580 ± 10100]	µg/g creat; mean ± SD [pg/mg creat; mean ± SD]
Maestri (Maestri 1997)	S-BMA	1997	<0.5 – 29.9 [<500-29900]	<0.5 – 32.7 [<500-32700]	µg/g creat; mean ± SD [pg/mg creat; mean ± SD]
B'Hym (B'Hymer 2011)	S-BMA	2011	10.5 ± 10.2	8.2 ± 7.8	ng/mL; mean ± SD
Pluym (Pluym 2015)	S-BMA	2015	2.3 (1.36-4.39) [2300 (1360-4390)]	3.1 (0.58-19.6) [3100 (580-19600)]	µg/g creat; median (min-max) [pg/mg creat; median (min-max)]
Alwis (Alwis 2012)	S-BMA	2012	16 ± 29	15 ± 32	ng/mL; mean ± SD

[1] Values converted to PMI's units are presented in [square brackets]
[2] Units uses in PMI's units are presented in [square brackets]

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Table 6 Representative Reductions of Levels of BoExp Achieved upon Smoking Cessation – Literature Review of Longitudinal Studies

Author	BoExp	Publication year	Duration of cessation	Levels at baseline [1]	Levels after cessation [1]	Units; measure of dispersion [2]
Frost-Pineda (Frost-Pineda 2008)	COHb	2008	8 days	6.8 ± 1.7	1.1 ± 0.2	%; mean ± SD
Ramos (Ramos 2011)	COHb	2011	180 days	1.52 ± 1.0	0.552 ± 0.30	%; mean ± SD
Tricker (Tricker 2012a)	COHb	2012	8 days	3.54 ± 1.5	0.48 ± 0.23	%; mean ± SD
Tricker (Tricker 2012b)	COHb	2012	8 days	5.53 ± 1.63	1.17 ± 0.32	%; mean ± SD
Polonia (Polonia 2009)	COHb	2009	6 months	5.9 ± 1.67	1.7± 0.56	%; mean ± SD
Tricker (Tricker 2012d)	COHb	2012	8 days	4.41 ± 1.38	0.45 ± 0.25	%; mean ± SD
Pezzuto (Pezzuto 2013)	COHb	2013	3 months	3.6 ± 1.5	2.1 ± 0.9	%; mean ± SD
Theophilus (Theophilus 2015)	COHb	2015	5 days	4.3 ± 1.2	1 ± 0.1	%; mean ± SD
Theophilus (Theophilus 2015)	COex	2015	5 days	22.9 ± 7.2	2.0 ± 0.7	ppm ± SD
Sarkar et al. (Sarkar 2008)	MHBMA	1008	8 days	2.70 ± 1.59 [2700 ± 1590]	0.09 ± 0.10 [90 ± 100]	µg/g creat [pg/mg creat]; mean ± SD
Carmella et al. (2009 and 2012) (Carmella 2012, Carmella 2009)	MHBMA	2009	3 days	33.05 ± 69.4 [7710.23 ± 16190.33]	2.71 ± 4.35 [632.22 ± 1014.81]	nmol/24h [ng/24h]; mean ± SD
Tricker et al. (Tricker 2012d)	MHBMA	2012	8 days	3.75 ± 3.15 [3750 ± 3150]	0.27 ± 0.14 [270 ± 140]	µg/24h [ng/24h]; mean ± SD
Tricker et al. (Tricker 2012a)	MHBMA	2012	8 days	1.63 ± 1.40 [1630 ± 1400]	0.28 ± 0.27 [280 ± 270]	µg/24h [ng/24h]; mean ± SD
Tricker et al. (Tricker 2012b)	MHBMA	2012	8 days	3.01 ± 3.22 [3010 ± 3220]	0.44 ± 0.32 [440 ± 320]	µg/24h [ng/24h]; mean ± SD
Tricker et al. (Tricker 2012c)	MHBMA	2012	5 days	2.51 ± 2.33 [2510 ± 2330]	0.36 ± 0.58 [360 ± 580]	µg/24h [ng/24h]; mean ± SD
Theopilus et al. (Theophilus 2015)	MHBMA	2015	5 days	5.8 ± 4.1 [5800 ± 4100]	0.5 ± 0.2 [500 ± 200]	µg/24h [ng/24h]; mean ± SD

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Author	BoExp	Publication year	Duration of cessation	Levels at baseline [1]	Levels after cessation [1]	Units; measure of dispersion [2]
Frost-Pineda et al. (Frost-Pineda 2008)	MHBMA	2008	8 days	4.7 ± 3.2 [4700 ± 3200]	0.22 ± 0.2 [220 ± 200]	µg/24h [ng/24h]; mean ± SD
Carmella (Carmella 2007)	3-HPMA	2007	4 weeks	3725 ± 2417 [824 ± 535]	719 ± 272 [159 ± 60]	pmol/mg creat [ng/mg creat]; mean ± SD
Frost-Pineda (Frost-Pineda 2008)	3-HPMA	2008	8 days	1834.3 ± 598.8	302.3 ± 94.7	µg/24h; mean ± SD
Carmella (Carmella 2009)	3-HPMA	2009	3 days	10020 ± 5150 [2217 ± 1140]	1336 ± 923 [296 ± 204]	nmol/24h [µg/24h]; mean ± SD
Tricker (Tricker 2012c)	3-HPMA	2012	5 days	1.37 ± 0.74 [1370 ± 740]	0.50 ± 0.20 [500 ± 200]	mg/24h [µg/24h]; mean ± SD
Tricker (Tricker 2012d)	3-HPMA	2012	8 days	1.91 ± 0.74 [1910 ± 740]	0.40 ± 0.23 [400 ± 230]	mg/24h [µg/24h]; mean ± SD
Tricker (Tricker 2012a)	3-HPMA	2012	8 days	2.61 ± 0.73 [2610 ± 730]	1.68 ± 0.37 [1680 ± 370]	mg/24h [µg/24h]; mean ± SD
Tricker (Tricker 2012b)	3-HPMA	2012	8 days	1.68 ± 0.97 [1680 ± 970]	0.48 ± 0.15 [480 ± 150]	mg/24h [µg/24h]; mean ± SD
Theophilus (Theophilus 2015)	3-HPMA	2015	5 days	2.4 ± 0.8 [2400 ± 800]	0.5 ± 0.2 [500 ± 200]	mg/24h [µg/24h]; mean ± SD
Feng et al. (Feng 2006)	S-PMA	2006	8 days	4.49 ± 4.29 [4490 ± 4290]	0.32 ± 0.26 [320 ± 260]	µg/24h [ng/24h]; mean ± SD
Sarkar et al. (Sarkar 2008)	S-PMA	2008	8 days	4.98 ± 2.89 [4980 ± 2890]	0.27 ± 0.05 [270 ± 50]	µg/g creat [pg/mg creat]; mean ± SD
Frost-Pineda et al. (Frost-Pineda 2008)	S-PMA	2008	8 days	6.0 ± 3.9 [6000 ± 3900]	0.4 ± 0.1 [400 ± 100]	µg/24h [ng/24h]; mean ± SD
Carmella et al. (Carmella 2009)	S-PMA	2009	3 days	3.20 ± 3.80 [765.76 ± 909.34]	0.396 ± 0.345 [94.76 ± 82.56]	nmol/24h [ng/24h]; mean ± SD
Tricker et al. (Tricker 2012c)	S-PMA	2012	5 days	3.75 ± 3.55 [3750 ± 3550]	0.53 ± 1.03 [530 ± 1030]	µg/24h [ng/24h]; mean ± SD
Tricker et al. (Tricker 2012d)	S-PMA	2012	8 days	4.72 ± 3.43 [4720 ± 3430]	0.22 ± 0.08 [220 ± 80]	µg/24h [ng/24h]; mean ± SD

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Author	BoExp	Publication year	Duration of cessation	Levels at baseline [1]	Levels after cessation [1]	Units; measure of dispersion [2]
Tricker et al. (Tricker 2012a)	S-PMA	2012	8 days	3.41 ± 1.88 [3410 ± 1880]	1.37 ± 0.42 [1370 ± 420]	µg/24h [ng/24h]; mean ± SD
Tricker et al. (Tricker 2012b)	S-PMA	2012	8 days	3.54 ± 0.34 [3540 ± 340]	0.34 ± 0.23 [340 ± 230]	µg/24h [ng/24h]; mean ± SD
Theophilus et al. (Theophilus 2015)	S-PMA	2015	5 days	0.9 ± 0.4 [900 ± 400]	0.4 ± 0.2 [400 ± 200]	µg/24h [ng/24h]; mean ± SD
Roethig (Roethig 2007)	Total NNAL	2007	8 days	NM	– 73 ± 13	% change from BL; mean ± SD
Frost-Pineda (Frost-Pineda 2008)	Total NNAL	2008	8 days	528.8 ± 221.7	117.8 ± 60.4	ng/24h; mean ± SD
Carmella (Carmella 2009)	Total NNAL	2009	3 days	2.70 ± 2.03 [564.95 ± 424.76]	0.935 ± 0.496 [195.64 ± 103.78]	nmol/24h [ng/24h]; SD
Stepanov (Stepanov 2009b)	Total NNAL	2009	2 weeks (NRT)	1.07 ± 0.8	0.15 ± 0.10	pmol/mL; mean ± SD
Stepanov (Stepanov 2009a)	Total NNAL	2009	2 weeks (NRT)	1.20 ± 0.688 [251.09 ± 143.96]	0.264 ± 0.166 [55.24 ± 34.73]	pmol/mg creat [pg/mg creat]; mean ± SD
Stepanov (Stepanov 2009a)	Total NNAL	2009	6 weeks (NRT)	1.20 ± 0.688 [251.09 ± 143.96]	0.154 ± 0.112 [32.22 ± 23.43]	pmol/mg creat [pg/mg creat]; mean ± SD
Tricker (Tricker 2012c)	Total NNAL	2012	5 days	221 ± 154	80 ± 69	ng/24h; mean ± SD
Tricker (Tricker 2012d)	Total NNAL	2012	8 days	231.0 ± 112.8	59.2 ± 31.4	ng/24h; mean ± SD
Tricker (Tricker 2012a)	Total NNAL	2012	8 days	146.34 ± 86.08	45.06 ± 37.82	ng/24h; mean ± SD
Tricker (Tricker 2012b)	Total NNAL	2012	8 days	237 ± 192	83 ± 82	ng/24h; mean ± SD
Theophilus (Theophilus 2015)	Total NNAL	2015	5 days	731 ± 399	311 ± 265	ng/24h; mean ± SD
Stepanov (Stepanov 2009a)	Total NNN	2009	56 days	0.140 ± 0.194 [24.81 ± 34.38]	0.080 ± 0.142 [14.18 ± 25.16]	pmol/mg creat [pg/mg creat]; mean ± SD
Urban (Urban 2009)	Total NNN	2009	5 days	3.1 ± 0.8	< LLOQ (2 pg/ml)	pg/mL; mean ± SD

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Author	BoExp	Publication year	Duration of cessation	Levels at baseline [1]	Levels after cessation [1]	Units; measure of dispersion [2]
Theophilus (Theophilus 2015)	Total NNN	2016	5 days	24.6 ± 26.3	4.0 ± 3.0	ng/24h; mean ± SD
Feng (Feng 2006)	Total 1-OHP	2006	8 days	0.14 ± 0.06 [140 ± 60]	0.04 ± 0.01 [40 ± 10]	µg/24h [ng/24h]; mean ± SD
Carmella (Carmella 2009)	Total 1-OHP	2009	3 days	1.36 ± 0.76 [296.82 ± 165.87]	0.83 ± 1.07 [181.15 ± 233.53]	nmol/24h [ng/24h]; mean ± SD
Tricker (Tricker 2012c)	Total 1-OHP	2012	5 days	136.7 ± 50.6	37.4 ± 11.2	ng/24h; mean ± SD
Tricker (Tricker 2012d)	Total 1-OHP	2012	8 days	191.4 ± 68.8	75.1 ± 54.2	ng/24h; mean ± SD
Tricker (Tricker 2012a)	Total 1-OHP	2012	8 days	236.02 ± 68.18	127.04 ± 24.71	ng/24h; mean ± SD
Tricker (Tricker 2012b)	Total 1-OHP	2012	8 days	174.7 ± 77.6	42.0 ± 15.3	ng/24h; mean ± SD
Frost-Pineda (Frost-Pineda 2008)	Total 1-OHP	2008	8 days	160.2 ± 82.5	41.1 ± 17.5	ng/24h; mean ± SD
Theophilus (Theophilus 2015)	Total 1-OHP	2015	5 days	313 ± 137	114 ± 53.4	mg/24h ; mean ± SD
Tricker (Tricker 2012c)	4-ABP	2012	5 days	10.4 ± 5.7	4.7 ± 1.6	ng/24h; mean ± SD
Tricker (Tricker 2012a)	4-ABP	2012	8 days	11.4 (7.1, 14.6)	5.5 (4.8, 7.0)	ng/24h; median (Q_{25}, Q_{75})
Tricker (Tricker 2012b)	4-ABP	2012	8 days	15.2 ± 8.6	4.6 ± 1.9	ng/24h; mean ± SD
Sarkar (Sarkar 2010)	4-ABP	2010	8 days	19.61 ± 10.67	2.25 ± 0.88	ng/24h; mean ± SD
Sarkar (Sarkar 2010)	4-ABP	2010	8 days (snus)	23.04 ± 10.43	2.91 ± 0.92	ng/24h; mean ± SD
Theophilus (Theophilus 2015)	4-ABP	2015	5 days	27.8 ± 7.9	5.5 ± 2.7	ng/24h; mean ± SD
Frost-Pineda (Frost-Pineda 2008)	4-ABP	2008	8 days	10.8 ± 16.5	2 ± 1.1	ng/24h; mean ± SD
Frost-Pineda (Frost-Pineda 2008)	2-NA	2008	5 days	16.1 ± 8.5	2.2 ± 0.9	ng/24h; mean ± SD
Tricker (Tricker 2012c)	2-NA	2012	8 days	7.5 ± 11.4	4.65 ± 1.57	ng/24h; mean ± SD
Tricker (Tricker 2012a)	2-NA	2012	8 days	33.5 (11.0, 46.6)	5.5 (4.8, 7.0)	ng/24h; median (Q25, Q75

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Author	BoExp	Publication year	Duration of cessation	Levels at baseline [1]	Levels after cessation [1]	Units; measure of dispersion [2]
Tricker (Tricker 2012b)	2-NA	2012	8 days	40.8 ± 45.1	5.9 ± 3.9	ng/24h; mean ± SD
Theophilus (Theophilus 2015)	2-NA	2015	5 days	36.4 ± 8.9	3.6 ± 1.4	ng/24h; mean ± SD
Frost-Pineda (Frost-Pineda 2008)	*o*-toluidine	2008	8 days	82.2 ± 54.8	40.9 ± 29.2	ng/24 h; mean ± SD
Sarkar (Sarkar 2010)	*o*-toluidine	2010	8 days	192 ± 75	54 ± 16	ng/24 h; mean ± SD
Tricker (Tricker 2012d)	*o*-toluidine	2012	8 days	169.8 ± 79.2	47.6 ± 24.4	ng/24h; mean ± SD
Tricker (Tricker 2012a)	*o*-toluidine	2012	8 days	70.53 ± 29.64	30.2 ± 8.35	ng/24h; mean ± SD
Tricker (Tricker 2012b)	*o*-toluidine	2012	8 days	111.5 ± 49.7	33.8 ± 23.6	ng/24h; mean ± SD
Tricker (Tricker 2012c)	*o*-toluidine	2012	5 days	91.1 ± 95.8	33.8 ± 29.0	ng/24h; mean ± SD
Theophilus (Theophilus 2015)	*o*-toluidine	2015	5 days	210 ± 63.5	96.6 ± 38.9	ng/24h; mean ± SD
Scherer et al. (Scherer 2014)	CEMA	2014	5 years	197 ± 101.3	1.3 ± 0.9	µg/24h; mean ± SD
Scherer et al. (Scherer 2010)	CEMA	2010	8 days	259.2 ± 117.6 (128.2–608.0)	25.7 ± 13.4 (14.9-75.5)	µg/24h; mean ± SD (range)
Theophilus et al (Theophilus 2015)	CEMA	2015	5 days	263 ± 83.4	38.3 ± 15.3	µg/24h; mean ± SD
Carmella (2009 and 2012) (Carmella 2012, Carmella 2009)	HEMA	2009, 2012	3 days	51 ± 47.1 [10570 ± 9761]	12 ± 16.8 [2487 ± 3482]	nmol/24h; mean ± SD [ng/24h]
Scherer (Scherer 2010)	HEMA	2010	8 days	25.8 ± 8.8 [25800 ± 8800]	6.79 ± 3.05 [6790 ± 3050]	µg/24 h [ng/24h]; mean ± SD
Theophilus (Theophilus 2015)	HEMA	2015	5 day	14.3 ± 9.6 [14300 ± 9600]	4.8 ± 2.2 [4800 ± 2200]	µg/24 h [ng/24h]; mean ± SD
Zhang (Zhang 2015)	3-OH-B[a]P	2015	8 days	2.83 ± 1.78 [2830 ± 1780]	1.30 ± 0.20 [1300 ± 200]	ng/g creat [fg/mg creat]; mean ± SD
Sarkar (Sarkar 2010)	3-OH-B[a]P	2010	8 days	162.70 ± 126.33	55.93 ± 21.71	pg/24h; mean ± SD
Theophilus (Theophilus 2015)	3-OH-B[a]P	2015	5 days	242.0 ± 138	67.8 ± 36.7	pg/24h; mean ± SD

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Author	BoExp	Publication year	Duration of cessation	Levels at baseline [1]	Levels after cessation [1]	Units; measure of dispersion [2]
Carmella (2009 and 2012) (Carmella 2012, Carmella 2009)	3-HMPMA	2009, 2012	3 days	9825 ± 1001 [2312 ± 236]	1325 ± 113 [312 ± 27]	nmol/24h; mean ± SD [converted into µg/24h]
Frost-Pineda (Frost-Pineda 2008)	3-HMPMA	2008	8 days	2695.7 ± 962	299 ± 166.3	µg/24h; mean ± SD
Scherer (Scherer 2007b)	3-HMPMA	2007	8 days	1890 ± 889	394 ± 200	µg/24 hours; mean ± SD
Theophilus (Theophilus 2015)	3-HMPMA	2015	5 days	715 ± 230	213 ± 131	µg/24h; mean ± SD

[1] Values converted to PMI's units are presented in [square brackets]
[2] Units used in PMI's clinical studies are presented in [square brackets]

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