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REVIEW ARTICLE

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From cultivation to cancer: formation of *N*-nitrosamines and other carcinogens in smokeless tobacco and their mutagenic implications

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ABSTRACT

Tobacco use is a major cause of preventable morbidity and mortality globally. Tobacco products, including smokeless tobacco (ST), generally contain tobacco-specific *N*-nitrosamines (TSNAs), such as *N*′-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK), which are potent carcinogens that cause mutations in critical genes in human DNA. This review covers the series of biochemical and chemical transformations, related to TSNAs, leading from tobacco cultivation to cancer initiation. A key aim of this review is to provide a greater understanding of TSNAs: their precursors, the microbial and chemical mechanisms that contribute to their formation in ST, their mutagenicity leading to cancer due to ST use, and potential means of lowering TSNA levels in tobacco products. TSNAs are not present in harvested tobacco but can form due to nitrosating agents reacting with tobacco alkaloids present in tobacco during certain types of curing. TSNAs can also form during or following ST production when certain microorganisms perform nitrate metabolism, with dissimilatory nitrate reductases converting nitrate to nitrite that is then released into tobacco and reacts chemically with tobacco alkaloids. When ST usage occurs, TSNAs are absorbed and metabolized to reactive compounds that form DNA adducts leading to mutations in critical target genes, including the *RAS* oncogenes and the p53 tumor suppressor gene. DNA repair mechanisms remove most adducts induced by carcinogens, thus preventing many but not all mutations. Lastly, because TSNAs and other agents cause cancer, previously documented strategies for lowering their levels in ST products are discussed, including using tobacco with lower nornicotine levels, pasteurization and other means of eliminating microorganisms, omitting fermentation and fire-curing, refrigerating ST products, and including nitrite scavenging chemicals as ST ingredients.

Abbreviations: AFB1: aflatoxin B_1; ALKBH2: alpha-ketoglutarate-dependent dioxygenase alkB homolog 2; AP: apurinic/apyrimidinic as in AP endonuclease; BaP: benzo[*a*]pyrene; BER: base excision repair; cAMP: cyclic AMP; CRP: cAMP receptor protein; CSA: Cockayne syndrome group A protein; CSB: Cockayne syndrome group B protein; DMSO: dimethylsulfoxide; DPC: DNA–protein crosslinks; GGR: global-genomic repair; Fe/S: iron/sulfur; MGMT: O^6-alkylguanine-DNA alkyltransferase (formerly O^6-methylguanine-DNA methyltransferase); MMR: mismatched repair; MPG: *N*-methylpurine-DNA glycosylase; NER: nucleotide excision repair; NMO: nitronate monooxygenase; NNAL: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-butanone; NNN: *N*′-nitrosonornicotine; NO_x: nitrous oxides; PARP-1: poly [ADP-ribose] polymerase 1; Polβ: DNA polymerase β; QH2: quinol; TCR: transcription-coupled repair; TSNAs: tobacco-specific *N*-nitrosamines; XPA: xeroderma pigmentosum group A protein; XRCC1: X-ray repair cross-complementing protein 1

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1. Smokeless tobacco and cancer

At the present mortality rate, the World Health Organization (WHO) predicts that during the twenty-first century, the worldwide deaths due to consumption of all types of tobacco products may reach *one billion*. Smokeless tobacco (ST) in its widely diverse forms, used orally or sometimes nasally, is consumed by nearly 356 million in 140 countries across all six WHO regions (Drope and Schluger 2018; Sinha 2018). A recent data analysis of 127 countries estimated the overall global ST-related disease burden, mainly cancer and ischemic heart disease, among adults results in an estimated 350,000 deaths each year. Among the regions of world, the highest ST disease burden prevalence (82%) is in the WHO South-East Asian Region (SEAR), especially in India and Bangladesh (Hatsukami et al. 2014). According to a recent Indian government monograph, 90% of all oral and pharyngeal cancers in India are caused by tobacco use, with 50% being associated with ST use (Gupta et al. 2016).

On a worldwide basis, a wide spectrum of ST products and ST preparations are made with various types of tobacco, cured and processed differently, that are combined with various chemicals and non-tobacco plant ingredients, and result in a wide range of carcinogenic tobacco-specific *N*-nitrosamine (TSNA) concentrations. The names and descriptions of commonly used ST products and ST preparations from different WHO regions of the world, their plant and chemical ingredients, mode of use (e.g. oral, nasal), and TSNA levels, if known, are presented in an appendix and chapters 3, 4, and 9 through 14 in a global ST report (Hatsukami et al. 2014). Additional products from the SEAR region, which has the highest level of ST use globally, and various fermented traditional snuffs, such as those made and used in Africa, have not been as well studied. When ST products are sucked, chewed, held in contact with oral tissues, or sniffed nasally, human exposure can occur, with nicotine, TSNAs, and other compounds being absorbed and entering circulation. TSNAs, which cause various types of cancer, are the main topic of this review (Hatsukami et al. 2014; Stanfill 2016; Bhisey and Stanfill 2016). A recent meta-analysis by Siddiqi et al. reported that in 2017 about 91,000 deaths occurring due to oral, pharyngeal, and esophageal cancer across the globe were caused by ST use (Siddiqi et al. 2020). In the United States, a significantly high risk of oral cancer is associated with chewing tobacco and snuff use (Wyss et al. 2016). Carcinogenic TSNAs contribute to the higher proportion of oral cancers attributable to ST product use in India and Sudan (50%) as compared to about 4% for the male population in the United States where the TSNA content is much lower (Idris et al. 1994, 1995; Boffetta et al. 2008; Bhisey 2012). In the SEAR and Eastern Mediterranean Region (EMR), use of ST products that may be manufactured or made in a cottage-industry setting, such as gutkha, khaini, zarda, mishri, tuibur, shammah, or toombak, or a hand-made ST preparation such as tombol or paan (betel quid with tobacco) (Hatsukami et al. 2014) have been associated with various other types of cancers of the oral cavity (especially the mouth, tongue, cheek, and gum), esophagus, pharynx, larynx, lung, stomach, and pancreas (Gupta et al. 2018). In particular studies of fermented cigar tobacco (Di Giacomo et al. 2007; Li et al. 2020) and certain ST products, especially Sudanese toombak, snuff have helped broaden our understanding of factors that contribute most to increased TSNAs formation during tobacco production (Idris et al. 1991, 1994, 1995, 1998; Wiernik et al. 1995; Wahlberg et al. 1999; Tyx et al. 2016, 2022; Smyth et al. 2017; Rivera et al. 2020; Rivera and Tyx 2021; Sami et al. 2021). Conversely, studies of snus, which is pasteurized and processed differently from other ST products, have highlighted effective means of minimizing the levels of TSNAs and other

carcinogens (Idris et al. 1998; Rutqvist et al. 2011; Lawler et al. 2020; Swedish Match 2023).

Among the 4200 chemicals present in ST, more than 30 are classified as known human carcinogens (group 1) by the International Agency for Research on Cancer (Boffetta et al. 2008; Hatsukami et al. 2014; IARC 2021). Group 1 carcinogens present in ST products include inorganic agents, such as metals or metalloids (e.g. arsenic, beryllium, cadmium, and nickel), that may be absorbed from the soil or deposited on tobacco leaves during cultivation (Golia et al. 2007; Pappas et al. 2008; Verma et al. 2010; Pappas 2011; Halstead et al. 2015). The molecular carcinogenicity of select metals has been reviewed recently; these agents are only briefly discussed in this review (Chen et al. 2019; Zhu and Costa 2020). Group 1 carcinogens also include organic compounds that can form reactive metabolites, then DNA adducts that can cause mutations. These carcinogens include polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene (BaP), and volatile organic compounds (VOCs), such as formaldehyde and acetaldehyde, that may be introduced during fire-curing; and aflatoxin B_1 (AFB1) produced by certain fungi. Areca nut, the fruit of the areca palm tree (*Areca catechu* Linn.), is a carcinogenic ingredient combined with tobacco in some ST products (e.g. gutkha, mawa, mainpuri; some zardas, or gul products) and in custom handmade ST preparations (e.g. paan, tombol, and dohra) used in India, Bangladesh, Pakistan, South and Southeast Asian nations, Southern China, some African, Middle Eastern, and Pacific Island nations; and worldwide among immigrants residing in numerous countries, including the U.S., U.K., Canada, and Australia. Besides its carcinogenicity, use of areca nut is also linked to a damaging oral malformation known as oral submucosal fibrosis (IARC 2004; Hatsukami et al. 2014; Gupta et al. 2018; Rao et al. 2020). Lastly, TSNAs, such as *N*′-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and their human metabolites formed by several biochemical and chemical mechanisms, which can lead to adducts and mutations, are a primary topic of this review (Section 2). Although numerous harmful substances (e.g. PAHs, VOCs, *N*-nitrosodimethylamine (NDMA), and other nitroso compounds, etc.) may be present in ST (Hatsukami et al. 2014), this present review focuses on a few IARC Group 1 carcinogens, including two TSNAs, namely NNN and NNK, but also, BaP, AFB1, and areca nut, all of which cause DNA adducts.

During certain types of curing that occur after harvesting, TSNAs can form chemically if nitrosating agents (e.g. reactive NO_x gases) react with tobacco alkaloids (Section 2.6) (Wahlberg et al. 1999; Di Giacomo et al. 2007; Fisher et al. 2012; Wang et al. 2017; Ma et al. 2019). Also, certain nitrate-reducing bacteria, if present and metabolically active, can play a role in nitrite production leading to TSNA formation, particularly during processing steps, such as fermentation, aging, and long-term storage of tobacco or products (Andersen et al. 1991; Wiernik et al. 1995; Wahlberg et al. 1999; Di Giacomo et al. 2007; Fisher et al. 2012; Ma et al. 2019). Nitrate-reducing bacteria can contain assimilatory (*nas*) or dissimilatory (*nar* and *nap*) nitrate reductase genes. The *nar* and *nap* genes encode respiratory nitrate reductase and periplasmic nitrate reductase, respectively. For clarity sake, in this review paper, our use of the term *nitrate-reducing bacteria* refers specifically to nitrate-reducing bacteria with dissimilatory nitrate reductases, including respiratory and periplasmic nitrate reductases, that can generate and release nitrite into tobacco. Indeed, bacterial dissimilatory nitrate reductases is a primary topic in Section 2 of this review (Wiernik et al. 1995; Wahlberg et al. 1999; González et al. 2006; Sparacino-Watkins et al. 2014; González et al. 2017).

This review also focuses on formation, bioactivity, and DNA-adduct mechanism of chemical carcinogenesis related to TSNAs during ST processing and when ST is introduced into the human body. It further provides an exploration of the molecular biology and biochemistry of nitrate reduction facilitated by certain nitrate-reducing bacteria and the chemistry of nitrosation occurring during processing of ST products but also human metabolism, adduct and mutation formation following ST usage. Biochemical and chemical aspects of DNA repair are also covered. Although knowledge gaps exist about the role of microorganisms in TSNA formation in ST products, more research is underway to fill these information gaps. Sections 2 and 3 presents a multi-disciplinary overview of the formation of TNSAs during ST production and their bioactivity once introduced into the human body due to ST usage. We start by exploring absorption of nitrate fertilizer and biosynthesis of alkaloids by tobacco plants, followed by the actions of bacterial nitrate reductases and/or chemical nitrosation occurring post-harvest, and end with human absorption from ST products followed by metabolism, adduct formation, DNA repair, and lastly the formation of mutations that lead to the progression of cancer (Figure 1). Very importantly, we review in Section 4 select technologies or approaches that have been successfully used to limit or minimize the formation or introduction of TSNAs and other carcinogens during ST cultivation or production.

2. Accumulation of *N*-nitrosamines and other carcinogens in tobacco

2.1. Tobacco cultivation and production steps

In preparation for a tobacco crop, growers consider genetic traits of different *Nicotiana* species, varieties, or cultivars, soil composition, seasonal weather patterns, pests, pathogens, fertilizer and agricultural chemical application rates, and also planning, harvesting, and curing methods, as well as other parameters. Similar to other tobacco products, ST production from seed-to-product generally proceeds through a number of stages that can include seed planting, field transplanting of seedlings, cultivation, harvesting, curing, processing, and packaging. Although cultivation practices vary globally, the growth of tobacco and its chemical content is influenced by plant genetics and metabolism, weather (e.g. temperature and rainfall), length of growing season, but also by the levels of plant nutrients and contaminants in the water, ambient air, soil, and soil amendments (e.g. fertilizers, added nutrients, and manure) present in the agricultural environment that can be absorbed by tobacco roots or deposited on tobacco leaf surfaces. During cultivation, viable organisms (e.g. insects, nematodes, fungi, bacteria, etc.) and viruses can reside in or on tobacco plants, and also soil and residues of agricultural chemicals (e.g. pesticides, fungicides, herbicides, etc.) can deposit and remain on leaf surfaces of harvested tobacco (Davis and Nielsen 1999; Wahlberg et al. 1999; Golia et al.

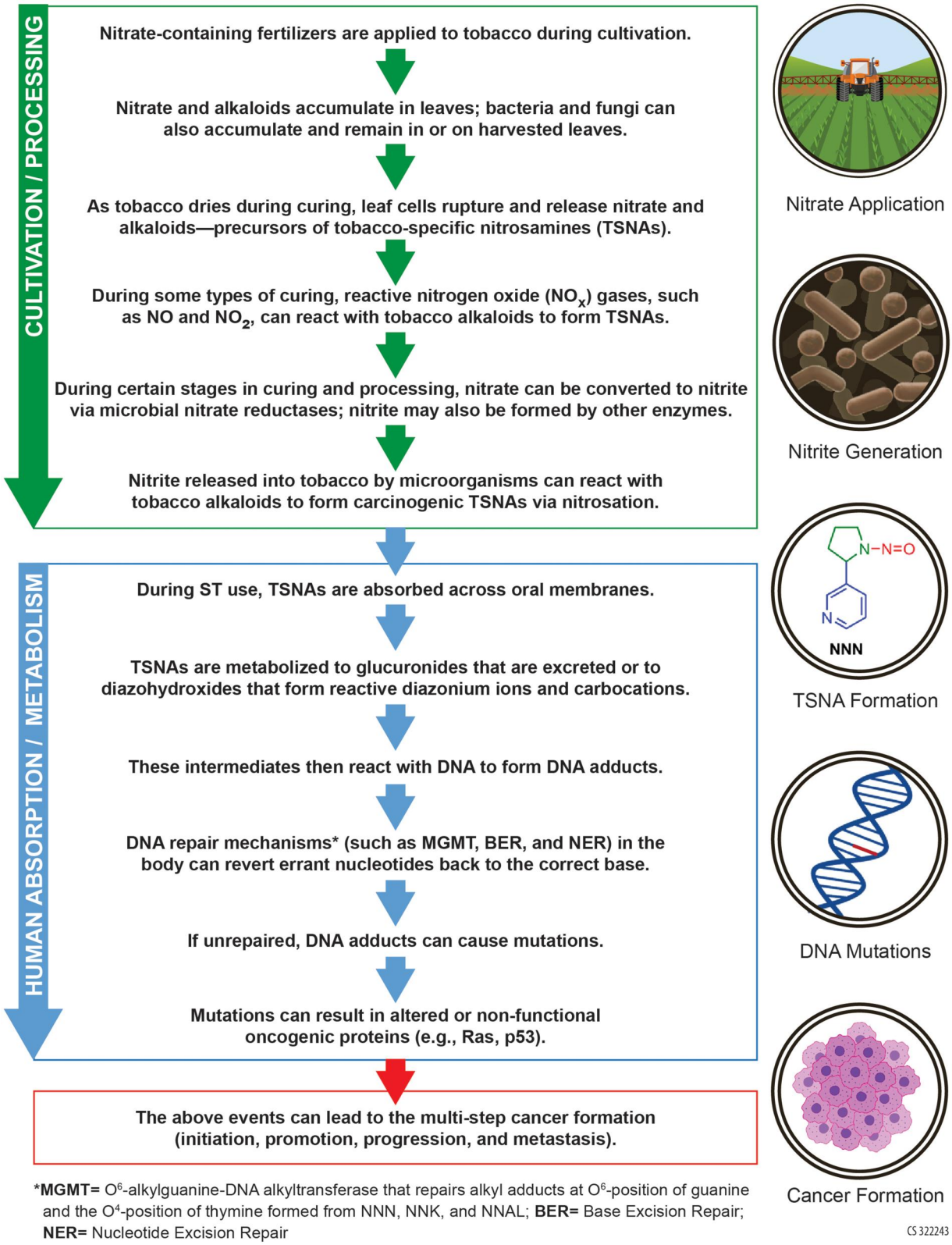


Figure 1. Cascade of physical, chemical, and biological events leading from cultivation to cancer formation. Green blocks are events that can occur in cultivation or processing. Blue boxes are those that occur during human absorption and metabolism. Key events addressed in this review are highlighted on the right. Additionally, the presence of certain fungi could produce and release nitrite and also mycotoxins, such as aflatoxin B_1, whereas, the use of fire-curing can result in the accumulation of polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene, and volatile organic compounds in tobacco.

2007; Rutqvist et al. 2011; Fisher et al. 2012; Chen et al. 2013; CORESTA 2013; Halstead et al. 2015; Dávila et al. 2020; Oldham et al. 2020; Rivera et al. 2020; Rivera and Tyx 2021; Sajid et al. 2021; Sami et al. 2021; Tobacco Guide 2023).

At harvest, individual leaves or entire stalks are excised from tobacco plants and are generally not washed; therefore, chemical residues and viable microorganisms on tobacco leaf surfaces may persist during production, and may be present

at some level in finished ST products (Wiernik et al. 1995; Davis and Nielsen 1999; Wahlberg et al. 1999; IFPA 2006; Golia et al. 2007; Larsson et al. 2008; CORESTA 2013; Han et al. 2016; Smyth et al. 2017; Rivera et al. 2020; Rivera and Tyx 2021; Sami et al. 2021; Tobacco Guide 2023). When harvesting is complete, tobaccos are dried by a method of curing, such as fire-, air-, flue-, or sun-curing, prior to further processing. Fire-curing is performed by hanging tobacco to dry in the heat and smoke produced by smoldering wood and sawdust fires maintained in the floor of a barn or building; unfortunately, carcinogenic PAHs (e.g. BaP) and VOCs (e.g. formaldehyde, acetaldehyde) are among the smoke-related chemicals that can be produced and accumulate on tobacco leaves during fire-curing. Moreover, the longer that tobacco is fire-cured, the more BaP and other PAHs can accumulate on the curing leaves. Air-curing involves air drying tobacco in a ventilated barn, whereas flue-curing involves blowing heated air across tobacco in an enclosed space. Sun-cured tobacco, which is heaped in piles in the field, laid on the ground, or laid or hung on racks, is dried in the sun (Davis and Nielsen 1999; Stepanov et al. 2008; Hearn et al. 2013; Hatsukami et al. 2014; Tobacco Guide 2023). Regardless of the curing method used, desirable curing endpoints include maximum production of tobacco leaf mass with minimal leaf damage caused by pests, pathogens, nutrient deficiencies, adverse weather, or other causes, but also appropriate characteristics, such as leaf color and thickness, aroma, and concentrations of nicotine and certain sugars. Although nicotine is a precursor of NNK and NNN, some of the nicotine in tobacco plants can also be converted via a plant enzyme to nornicotine, which is also a precursor of NNN. Although tobaccos used to make ST almost always contain nicotine, tobaccos with lower nornicotine levels are generally preferred to tobaccos with higher nornicotine levels (Davis and Nielsen 1999; Hatsukami et al. 2014; Lewis 2019; Tobacco Guide 2023).

In terms of tobacco processing and ingredients, ST varies globally from products that contain cured tobacco with little or no additives to complex products that are highly processed and have numerous chemical and non-tobacco plant ingredients (Hatsukami et al. 2014). TSNAs are often present at the end of curing, but can increase further during other steps. The tobacco used to make some products (e.g. khaini, toombak, moist snuff, dry snuff, traditional snuffs, etc.) may be subjected to one or more processing steps, including fermentation, aging, and long-term storage. These processing steps are often characterized by limited O_2 levels, increased microbial activity, and rapid chemical transformations, including the formation of nitrite and TSNAs. Processing of other types of ST products may omit all of these steps (Andersen et al. 1991; Brunnemann and Hoffmann 1991; Wiernik et al. 1995; Wahlberg et al. 1999; Di Giacomo et al. 2007; Rutqvist et al. 2011; Fisher et al. 2012; Hatsukami et al. 2014; Wang et al. 2017; Li et al. 2020). Tobacco processing may also include cutting, grinding, pulverizing, compressing, boiling, roasting, toasting, or pyrolyzing tobacco; increasing or decreasing moisture content; and blending of different types of tobacco (e.g. air- and fire-cured tobacco in snuff). Processing can also include the addition of chemical agents (e.g. flavorings, colorants, and salts), non-tobacco plant ingredients (e.g. areca nut, catechu, and spices), and pH-boosting agents (e.g. slaked lime, plant/fungal ashes, metallic carbonates, or other alkaline agents) that generally enhance oral absorption of nicotine and areca nut related compounds. At the end of production, tobaccos and ingredients are mixed and packaged to generate the final ST products (Stanfill et al. 2011, 2018; Lawler et al. 2013; Hatsukami et al. 2014; Nasrin et al. 2020).

Unlike other ST products, some snus products are produced using air-cured tobacco that is pasteurized to eliminate microorganisms. Production of some snus products can include the addition of food-grade ingredients and omission of steps such as fire-curing, fermentation, and aging. In addition to pasteurization, certain snus products are refrigerated where these products are sold. Refrigeration of ST products inhibits microbial growth and activity, and slows reactions that form TSNAs. Nitrite-scavenging chemicals, which are added to some snus products, capture nitrite to prevent its reaction with tobacco alkaloids to form TSNAs. These processing modifications in snus production tend to minimize the levels of TSNAs that can remain elevated in other ST product types that are processed differently (Hoffmann et al. 1994; Idris et al. 1998; Rundlöf et al. 2000; Rutqvist et al. 2011; Hatsukami et al. 2014; Lawler et al. 2020; Swedish Match 2023).

In terms of packaging, cottage industry or custom-made ST products or ST preparations (e.g. paan), once made, are often wrapped or enclosed in commonly available materials (e.g. cellophane, newspaper, foil, paper bags, and zip-lock bags). Manufactured ST products, on the other hand, usually contain tobacco material sealed inside more substantial packaging materials (e.g. metal, plastic, or cardboard containers, tearable or resealable foil packs, shrink wrapping, sealed plastic bags) with recognizable product names, brand names, colors, and graphics. Some types of tightly packed or tightly sealed packaging retain moisture but may limit O_2 levels in the product contents. After production, some product packages may contain microorganisms that produce nitrite in the time intervals between ST packaging and product sale while in various environments (e.g. in storage facilities, delivery vehicles, stores, street stalls, vending carts, etc.) and in ST packages between purchase and ST use. During these periods, the varying physical and chemical conditions (e.g. temperature, moisture, pH, nitrate, and O_2 levels) inside of some ST packages, may be amenable to nitrite generation and release by certain microorganisms. Moreover, conditions inside some ST packages may also allow nitrite, released from certain microorganisms into tobacco, to initiate the abiotic nitrosation reactions with tobacco alkaloids that generate TSNAs. In this review, the enzymatic and chemical steps that can lead to TSNA formation are discussed in detail (Andersen et al. 1991; Hoffmann et al. 1994; IARC 2004; Di Giacomo et al. 2007; Rutqvist et al. 2011; Hatsukami et al. 2014; Han et al. 2016; Jain et al. 2017; Smyth et al. 2017; Stanfill et al. 2018; Gunjal et al. 2020; Nasrin et al. 2020; Rao et al. 2020; Rivera and Tyx 2021).

During some types of curing, formation of TSNAs, including NNN and NNK, can occur due to nitrosation reactions when nitrosating agents react with tobacco alkaloids (Section 2.6). Another pathway to TSNAs, which is a primary topic in Section 2, involves two types of chemical precursors: nitrate absorbed into tobacco roots from nitrate-fertilized soils, and certain alkaloids (e.g. nicotine, nornicotine) naturally synthesized in

tobacco; and also certain microorganisms that generate and release nitrite (i.e. a reactive nitrosating agent) into tobacco that actually initiates TSNA formation. These chemical agents and microorganisms can naturally accumulate in or on tobacco leaves by the end of cultivation of nitrate-fertilized tobacco. Processing steps such as fermentation and aging are amenable to active microbial proliferation and nitrite formation (Figure 2). In a two-step process, TSNAs can be formed–first, certain microorganisms facilitate enzymatic conversion of nitrate to nitrite, or nitrite generated by other nitrite-producing enzymes, then the nitrite that is produced is released into tobacco (Section 2.5), then second, nitrosation reactions occur between nitrite and certain tobacco alkaloids to produce NNN and NNK (Section 2.6). These steps start late in curing and proceed through processing while appropriate conditions persist (Figure 2). When adequate precursors and particular microorganisms are present, this same two-step process described above can continue inside of ST products even after purchase, especially in those with higher moisture content and elevated temperatures (Andersen et al. 1991; Hoffmann et al. 1994; Wiernik et al. 1995; Wahlberg et al. 1999; Staaf et al. 2005; Di Giacomo et al. 2007; Rutqvist et al. 2011; Hatsukami et al. 2014; Tobacco Guide 2023). Next, we discuss the origins of the chemical precursors of TSNAs in tobacco during cultivation.

2.2. Uptake and utilization of nitrogen ions and soil constituents

Nitrogen uptake from the soil, including that provided by nitrate fertilizers, is vitally important to tobacco plants. As a tobacco plant grows, available nitrogen ions in the soil, such as nitrate and ammonium, are absorbed by the roots and are subsequently used to synthesize tobacco-specific molecules, such as tobacco alkaloids nicotine and nornicotine, but also biomolecules, such as amino acids, proteins, nucleotides, DNA, RNA, and chlorophyll, required for normal plant growth (Davis and Nielsen 1999; Garrett and Grisham 2016; Taiz et al. 2022). At the beginning of the growing season, tobacco leaves generally contain low levels of nitrate, even lower levels of nitrite, and essentially no TSNAs (Idris et al. 1991; Wiernik et al. 1995; Davis and Nielsen 1999; Law et al. 2016; Xu et al. 2020; Tobacco Guide 2023). In order to increase soil nitrate levels during tobacco cultivation, various nitrate-containing agents such as ammonium nitrate, calcium nitrate, potassium nitrate, sodium nitrate, or calcium ammonium nitrate can be applied as fertilizers. Alternatively, urea or ammonia, which do not contain nitrate, do not contribute to increased soil nitrate levels if used as fertilizers. Because nitrate anions naturally leach out of soils, reapplication of nitrate-containing fertilizers is required throughout the tobacco growing season to maintain appropriate soil nitrate levels (Davis and Nielsen 1999; Soares et al. 2012; Kaiser et al. 2015; Li et al. 2017; Tobacco Guide 2023).

The nitrate-acquiring process in plants includes root uptake, root-to-shoot transport, storage, and reduction of nitrate to nitrite and then to ammonium that is readily incorporated into numerous biomolecules (Tischner and Kaiser 2007). A number of specific transporter proteins facilitate nitrate uptake from the soil into root epidermal cells, which then moves layer by layer through the root cortex, endodermis, pericycle, and parenchyma, and finally into the vascular xylem that transports nitrate anions upwards to the leaves where it is stored in leaf cell vacuoles (Wang et al. 2012). Absorbed ammonium can be directly incorporated into nitrogen-containing compounds, whereas absorbed nitrate (NO_3^-) must first be metabolized to nitrite (NO_2^-), then rapidly to ammonium (NH_4^+) prior to further assimilation (Davenport et al. 2015). Complex and sophisticated biochemical mechanisms maintain nitrite (NO_2^-) at low levels in tobacco leaves. Lastly, nitrate (NO_3^-), which is not assimilated during the growing season, remains in harvested tobacco leaves and is a precursor in TSNA formation. In unburned tobacco and ST products, nitrate can reach as high as mg/g concentrations (Davis and Nielsen 1999; Stepanov et al. 2005; Henry et al. 2019; Mocniak et al. 2023; Tobacco Guide 2023).

In addition to the uptake of nitrogen ions, certain macronutrients (e.g. Ca, Mg, and S) and micronutrients (e.g. Cu, Fe, and Mo), required for plant growth and enzyme functioning, are absorbed from the soil by roots and are present in harvested leaves (Taiz et al. 2022). In some cases, it is necessary for substances containing S and Mo to be applied to promote normal tobacco growth during cultivation (Tobacco Guide 2023). Some metals or metalloids that are toxic (e.g. Al, Cr, and Hg) or carcinogenic (e.g. As, Be, Ni, Co, Cd, and Pb) can be present in contaminated soil or the agricultural environment. These harmful agents may be absorbed and translocated into tobacco leaves or may be deposited onto tobacco leaf surfaces by some means during cultivation or harvesting (Golia et al. 2007; Pappas et al. 2008; Verma et al. 2010; Pappas 2011; Halstead et al. 2015). During curing, tobacco dries and its leaf cells rupture, releasing nutrients (Wahlberg et al. 1999), including S, Fe, and Mo, that can be utilized by bacteria in assembling enzyme cofactors (i.e. heme, Fe/S clusters, and Mo centers) necessary for nitrate reduction activity (Section 2.4) and in assembling Fe/S clusters present in transcriptional regulators (e.g. ArnR) necessary in sensing NO_3^- and O_2 levels to express bacterial *nar* genes (Figure A1) (Chandrangsu et al. 2017).

2.3. Alkaloid biosynthesis in tobacco

Worldwide, at least 83 different *Nicotiana* species have been identified, which possess various metabolic pathways producing different concentrations of tobacco alkaloids that can form TSNAs, including nicotine, nornicotine, anabasine, and anatabine; however, we will focus only on the first two compounds that are precursors to the most potent TSNA carcinogens, NNN and NNK (Davis and Nielsen 1999; Hatsukami et al. 2014; Berbeć and Doroszewska 2020; Tobacco Guide 2023). Among the *Nicotiana* species, it is *N. tabacum* (i.e. cultivated tobacco), with its numerous cultivars that have different characteristics and varying levels of nicotine and nornicotine, that is the most common species used to make ST products worldwide. Another species, *N. rustica* (i.e. Aztec tobacco), used to make ST products in South America (e.g. rapé), Sudan (e.g. toombak), India, Bangladesh, and other Asian countries (e.g. zarda, khaini, gul, sada pata, chewing tobacco, maras, etc.) can contain even higher levels of nicotine and nornicotine than found in *N. tabacum*. Lastly, the leaves of *N. glauca* (i.e. tree tobacco), which are chewed or occasionally

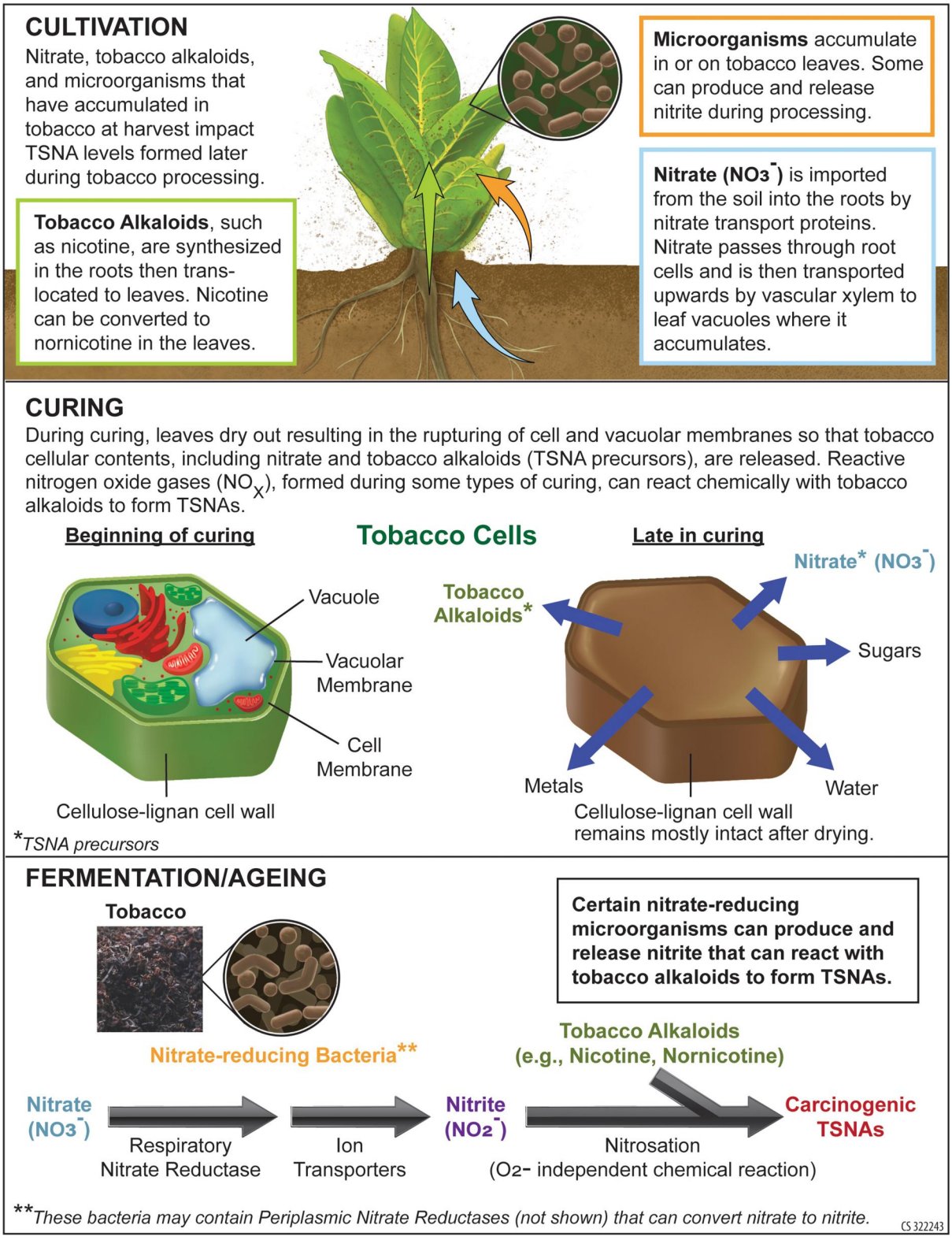


Figure 2. Key agricultural and processing steps contributing to the formation of TSNAs in nitrate-fertilized tobacco by nitrate reduction and nitrosation. If present, bacteria with other nitrite-producing enzymes (e.g. nitronate monooxygenase) or certain fungi with nitrate reductases could produce and release nitrite at some point in tobacco processing.

used to make ST products, contain little or no nicotine but elevated levels of anabasine that is toxic and sometimes lethal (Idris et al. 1991; Sinha 2004; Stepanov et al. 2005; Furer et al. 2011; Stanfill et al. 2011, 2015, 2018; Lisko et al. 2013; Hatsukami et al. 2014). The physiological role that tobacco alkaloids play in tobacco and the metabolic pathways and biological processes that lead to nicotine and nornicotine being present in tobacco leaves at harvest are summarized below.

All tobacco species are members of the nightshade plant family (i.e. *Solanaceae* family) and some synthesize pyrrolidine–pyridine alkaloids that act as a chemical defense to ward off herbivores and pathogens that can damage tobacco. The

tobacco alkaloid nicotine, which is produced in the roots then translocated to tobacco leaves and is present in hair-like trichomes on the leaf surfaces, exerts its insecticidal properties by binding to acetylcholine receptors in insects exposed to it (Baldwin 1989; Steppuhn et al. 2004; Benowitz et al. 2009; Cui et al. 2011; Martínez-Jarquín et al. 2018; Zenkner et al. 2019; Kanmani et al. 2021; Sahu et al. 2022). Tobacco biosynthesis of nicotine and nornicotine begins when nitrate or ammonia are absorbed via tobacco roots and then assimilated into one of three amino acids (arginine, ornithine, and aspartic acid). In one biosynthetic pathway, arginine or ornithine is converted to putrescine, which is further transformed by several enzymatic steps into a reactive intermediate, *N*-methylpyrrolinium cation, which contains a pyrrolidine ring. In a separate pathway, aspartic acid undergoes enzymatic steps resulting in 3,6-dihydronicotinic acid, which contains a dihydropyridine ring. Finally, nicotine, a pyrrolidine–pyridine alkaloid, is generated when a *N*-methylpyrrolinium cation reacts with 3,6-dihydronicotinic acid in root cortical cells (Shi et al. 2006; Shoji and Hashimoto 2013; Uriarte et al. 2017; Lewis 2019; Zenkner et al. 2019). Later in tobacco cultivation, a jasmonate-inducible alkaloid transporter facilitates translocation of nicotine from the tobacco roots to the leaves. Once in the leaves, a certain percentage of nicotine molecules can be converted to nornicotine in tobacco plants that contain the nicotine *N*-demethylase enzyme, which removes the methyl group from the pyrrolidine ring in nicotine and substitutes it with a hydrogen to yield nornicotine. At harvest, tobacco alkaloids may be present in tobacco leaves or on their surfaces (Morita et al. 2009; Cui et al. 2011; Lisko et al. 2013; Zenkner et al. 2019; Tobacco Guide 2023). Besides their bioactivity, tobacco alkaloids, such as nicotine and nornicotine, can chemically react with nitrite, generated and released by certain microorganisms, to form the most carcinogenic TSNAs in ST products, NNN and NNK (Wahlberg et al. 1999; Shi et al. 2006; Shoji and Hashimoto 2013; Li and Hecht 2022). The introduction of microorganisms into tobacco and the activity of certain microbial enzymes, which can form nitrite leading to TSNA formation in ST products, are covered next.

2.4. Presence of bacteria and fungi in tobacco

Microorganisms, present in soil and agricultural environments, can be transferred to tobacco during cultivation and may remain in tobacco tissues or on tobacco leaf surfaces at the end of cultivation (Wiernik et al. 1995; Wahlberg et al. 1999; Golia et al. 2007; Chang and Parsonnet 2010; Pauly and Paszkiewicz 2011; Rivera et al. 2020; Rivera and Tyx 2021; Sami et al. 2021). During harvesting, tobacco is sometimes laid directly on the soil briefly. Tobacco used to make toombak snuff is sun-cured in the tobacco field. In that type of curing, tobacco is heaped in piles in the field and remains for as long as 45 days in close proximity to soil. Hence, microorganisms can be introduced into tobacco during cultivation, harvesting, or curing, but also due to contact with bare hands occurring during harvesting and handling of tobacco, or during hand-mixing of the final ST products (Idris et al. 1991, 1998; Smyth et al. 2017). During later stages of curing, tobacco dries out and cellular membranes rupture, thus releasing cell contents, such as nitrate, alkaloids, and sugars, that can be subjected to microbial metabolism, including nitrite production, and chemical reactions leading to TSNAs (Figure 2). During fermentation and aging of tobaccos for ST and cigars, bacterial communities are influenced by pH, temperature, moisture, and oxygen availability (Andersen et al. 1991; Wahlberg et al. 1999; Di Giacomo et al. 2007; Fisher et al. 2012). Other potential sources of microorganisms in ST products may include processing environments or equipment (e.g. fermentation vats, mixing, or storage containers), non-food grade ingredients (Idris et al. 1991; Wahlberg et al. 1999; Rutqvist et al. 2011; Fisher et al. 2012; Smyth et al. 2017), or those intentionally added to aid fermentative processes (Fisher et al. 2012).

Even with the conditions encountered during ST production, some bacteria survive and remain viable in ST products (Han et al. 2016; Smyth et al. 2017). Indeed, the bacterial microbiota in various tobacco products, such as cigarettes, cigars, and various ST types, is highly diverse and has been studied extensively in recent decades (Di Giacomo et al. 2007; Sapkota et al. 2010; Han et al. 2016; Law et al. 2016; Tyx et al. 2016; Al-Hebshi et al. 2017; Chopyk et al. 2017; Smyth et al. 2017; Rivera et al. 2020; Rivera and Tyx 2021; Sajid et al. 2021; Sami et al. 2021; Tyx et al. 2022). Certain bacteria found in ST products can contribute to TSNAs. Many ST products can contain bacteria with assimilatory and/or dissimilatory nitrate reductase genes (Law et al. 2016; Tyx et al. 2016, 2022; Rivera et al. 2020; Rivera and Tyx 2021). *Enteractinococcus*, *Corynebacterium*, and *Staphylococcus* are among the nitrate-reducing species found in some ST products that contain genes in the *nar* operon encoding respiratory (dissimilatory) nitrate reductase. Other ST products, such as dry snuff, also contain members of the *Enterobacteriaceae* family that contain genes in the *nap* operon that encode periplasmic nitrate reductases; these bacteria may or may not contain *nar* genes (Tyx et al. 2016, 2022; Smyth et al. 2017; Rivera et al. 2020; Rivera and Tyx 2021; Sami et al. 2021). Nitronate monooxygenase, which combines O_2 with various nitroalkanes to form certain aldehydes (e.g. acetaldehyde) and nitrite, has been found among the microorganisms in some ST products (Gadda and Francis 2010; Rivera et al. 2020; Torres-Guzman et al. 2021). However, further investigation is needed to improve our understanding of the pathways critical in the formation of TSNAs among various ST product types, which vary in their chemical and microbiological content.

While some fungi also have dissimilatory nitrate reductases (Shoun et al. 2012) and were found to play a role early in the fermentation of tobacco (Di Giacomo et al. 2007), fungal populations have not been studied as extensively as the bacterial populations and may only be present in low abundances in ST products at the time of purchase (Rivera et al. 2020). A deeper study of fungi in this area of tobacco product research will provide greater understanding of their role in the formation of TSNAs and other carcinogens (e.g. aflatoxins and other mycotoxins) in ST products. In Section 2.5, discussion related to TSNA formation focuses primarily on bacterial dissimilatory nitrate reductases (Wiernik et al. 1995; Wahlberg et al. 1999; Law et al. 2016; Tyx et al. 2016; Rivera et al. 2020; Rivera and Tyx 2021).

2.5. Bacterial nitrate reduction proteins involved in generation and release of nitrite

2.5.1. Bacterial nitrogen ion transport proteins

In order for nitrate present in tobacco to enter the cytoplasm of bacterial cells and for nitrite that is formed to exit, a transmembrane ion transporter is required. Some nitrate-reducing bacteria with *nar* operons, such as *narKGHJI* and *narGHJIK*, produce NarK, a nitrate:nitrite antiporter. The NarK transporter is thought to operate by a "rocker switch" mechanism in which nitrate enters and is transferred to the cytoplasmic side before nitrite is transferred outward to the periplasmic side (Yan et al. 2013). Researchers have found another nitrate: nitrite antiporter in *Staphylococcus* species annotated as NarT (Fast et al. 1996). Some bacteria may contain other nitrogen ion transporters (e.g. NrtABC) (Frías et al. 1997) and/or porins, barrel-shaped proteins in the outer membrane, that facilitate passive transport that allows nitrogen ions to enter the periplasm or exit to the extracellular environment (Cowan et al. 1995). When nitrate enters bacterial cells through transporters or porins, it can be metabolized to nitrite by dissimilatory nitrate reductases localized in the cytoplasm or periplasm. Once nitrite is formed, it can exit through transporters and/or porins and cause extracellular nitrite to accumulate (Richardson 2000; González et al. 2006, 2017). Section 2.5.2 delves into the molecular biology of *nar* and *nap* genes and the protein biochemistry related to the structure and functioning of bacterial nitrate reductase proteins and their molybdenum-containing active sites where nitrite-producing reactions key to TSNA formation can occur.

2.5.2. Bacterial dissimilatory nitrate reductases

As mentioned already, TSNAs can be formed when tobacco alkaloids react with nitrogen oxide (NO_x) gases during curing but also via nitrite-catalyzed nitrosation. Nitrite is commonly produced by dissimilatory nitrate reductases, which are molybdoenzymes. Because nitrate-reducing bacteria with respiratory nitrate reductases or periplasmic nitrate reductases are thought to play a role in TSNA formation, we present information about the structure and function of these enzymes (Wiernik et al. 1995; Wahlberg et al. 1999; Law et al. 2016; Tyx et al. 2016, 2022; Smyth et al. 2017; Rivera and Tyx 2021). Nitrate reductases contain a single molybdenum atom per active site and are the only metalloenzymes currently known to catalyze the nitrate reduction reaction, which converts nitrate to nitrite (González et al. 2006, 2017; Maia et al. 2017).

Nitrate-reducing bacteria can contain one or more of three types of nitrate reductases that have distinct structure, subcellular location, and physiological roles; however, all three types of nitrate reductases catalyze the same oxotransferase reaction and contain a molybdenum-dependent catalytic subunit that contains two redox-active cofactors: the molybdopterin cofactor and one 4Fe–4S cluster (González et al. 2006, 2017; Maia et al. 2017). Prokaryotic nitrate reductases are classified as: (1) assimilatory nitrate reductases (encoded in *nas* genes), which are cytoplasmic enzymes key in nitrogen assimilation and subsequent biosynthetic metabolism, yet do not produce nitrite that is exported; (2) respiratory nitrate reductases (*nar*) that are membrane-bound and face the cytoplasm; and (3) periplasmic nitrate reductases (*nap*) that are located on the outside of the inner membrane in the periplasmic space. Assimilatory nitrate reductase (*nas*) reduces NO_3^- to NO, N_2O, N_2, or NH_4, whereas dissimilatory nitrate reductases encoded in *nar* and *nap* genes do not incorporate NO_3^- into biomass but convert it to NO_2^- that is released into tobacco (González et al. 2006, 2017; Sparacino-Watkins et al. 2014; Maia et al. 2017).

Respiratory nitrate reductases (*nar*), often expressed under low O_2 conditions, are typically involved in energy generation, detoxification, and redox regulation (Maia et al. 2017). Certain stages in tobacco processing (e.g. fermentation, aging) may provide low O_2 conditions and available NO_3^- (Andersen et al. 1991; Di Giacomo et al. 2007; Fisher et al. 2012) conducive to *nar* gene expression. Regulatory systems in certain bacteria control *nar* genes so that nitrate respiration occurs appropriately in response to O_2 and NO_3^-. For example, transcriptional regulators such as ArnR and GlxR control *nar* gene expression (Figure A1). Under aerobic conditions, a FeS cluster bound form of ArnR (i.e. FeS-ArnR) is associated with the promoter region and prevents *nar* operon expression (Nishimura et al. 2007, 2008, 2011, 2014; Madeira et al. 2019). In the presence of NO_3^- and anaerobic conditions, FeS-ArnR loses the FeS cluster, resulting in ArnR being released from the promoter, thus allowing for *nar* operon expression. The *nar* operon also requires an activator, GlxR, that binds to the *nar* operator region and promotes *nar* gene expression in response to cAMP due to low O_2 conditions (Nishimura et al. 2008, 2011, 2014). The ArnR/GlxR regulatory system and nitrogen regulatory elements (e.g. NreABC) are discussed in more detail in Appendix A1.

Some bacterial *nar* operons (e.g. *narKGHJI*, *narGHJIK*) contain the blueprint for nitrate/nitrite transporters, the respiratory nitrate reductase (NarGHI), and the NarJ chaperone protein that aids in the assembly of the three reductase subunits and its active site in a finely orchestrated process, shown in Figure A2 (Blasco et al. 1998; Lanciano et al. 2007; Bay et al. 2015). Some *Staphylococcus* species contain a gene for a NarT ion transporter (Fast et al. 1996) and a separate *narGHJI* operon (Maia et al. 2017). Genes in the molybdenum cofactor biosynthesis pathway are also expressed to synthesize components of the active site (Palmer et al. 1996; Vergnes et al. 2004). The respiratory nitrate reductase complex (NarGHI), which is cytoplasm facing, is a dimer of heterotrimers $(\alpha\beta\gamma)_2$ consisting of two copies each of the α-, β-, and γ-subunits, also denoted as NarG, NarH, and NarI, respectively (Bertero et al. 2003; González et al. 2006, 2017; Maia et al. 2017) (Figure 3(A)).

In terms of the individual protein subunits, NarG is the catalytic subunit that is cytoplasm-facing and holds a [4Fe–4S] cluster and the molybdenum-containing active site where nitrate is reduced to nitrite (Figure 4). Moreover, the electron-conducting subunit NarH holds one [3Fe–4S] and three [4Fe–4S] centers that convey electrons from NarI to NarG. Lastly, NarI proteins anchor NarGHI to the cytoplasmic side of the inner membrane of a bacterium. NarI also holds two b-type hemes that catalyze quinol oxidation and releases protons to the periplasmic space, which boosts the proton

gradient. When assembled and functioning, NarGHI facilitates electron flow from quinol to nitrate that subsequently generates a proton gradient necessary for ATP synthesis but produces nitrite as a by-product. Transporters (e.g. NarK, NarT, and NrtABC) or porins (Section 2.5.1) allow nitrate to enter and nitrite to exit the bacterial cell (Bertero et al. 2003; González et al. 2006, 2017; Maia et al. 2017).

Periplasmic nitrate reductases (Figure 3(B)) are functionally diverse and play a role in dissimilatory nitrate reduction (Cruz-García et al. 2007; Tamegai et al. 2007), but also the maintenance of cellular redox potential (Richardson 2000) and nitrate scavenging (Potter and Cole 1999); thus, periplasmic nitrate reductases also present another potential mechanism by which nitrite can be produced and released into tobacco (Law et al. 2016; Tyx et al. 2016, 2022; Sami et al. 2021). Similar to the structure of the NarG subunit, the active site of the NapA subunit is buried ~15Å below the protein's surface. To reach the active sites of these reductases, nitrate anions pass through a large funnel-shaped cavity to reach the molybdenum center at the catalytic core, where nitrate is reduced and nitrite is generated and subsequently released (Sparacino-Watkins et al. 2014) (Figures 3(A,B) and 4).

Although the content and order of genes differ, all *nap* operons contain the *napA* gene for the catalytic subunit and many have *napB* that encodes the electron-conducting subunit that makes up the NapAB heterodimer found in the periplasm of some bacteria. Genes in *nap* operons that have been identified are thought to function in the maturation of catalytic subunits (*napF*, *napL*, and *napD*) or in electron transfer (*napB*, *napC*, *napG*, and *napH*) with NapA, whereas the function of *napE* is not well described (Sparacino-Watkins et al. 2014). Among proteobacteria, electrons are provided to cytoplasmic NapAB via periplasm-facing NapC and/or NapGH associated with the inner membrane as shown in Figure A3. The *nap* operon structure, organization, and *nap* gene content among bacteria are highly variable, with no consensus among phylogenetic classes. Diverse *nap* operons are found among soil bacteria, such as betaproteobacteria (e.g. *napEDABC*), gammaproteobacteria, such as the *Enterobacteriaceae* family (*napFDABC*, *napDABC*, and *napFDAGHBC*) and the *Pseudomonadaceae* family (*napEDABC* and *napEFDABC*), and epsilonproteobacteria (*napAGHBFLD*), but each contain *napC* and/or *napGH* genes involved in electron transfer. Individual bacteria can have more than one *nap* operon with different organization (González et al. 2006; Simpson et al. 2010; Hartsock and Shapleigh 2011; Sparacino-Watkins et al. 2014). Members of these taxonomic groups mentioned above have been found among tobacco and tobacco-containing products (Wiernik et al. 1995; Wahlberg et al. 1999; Huang et al. 2010;

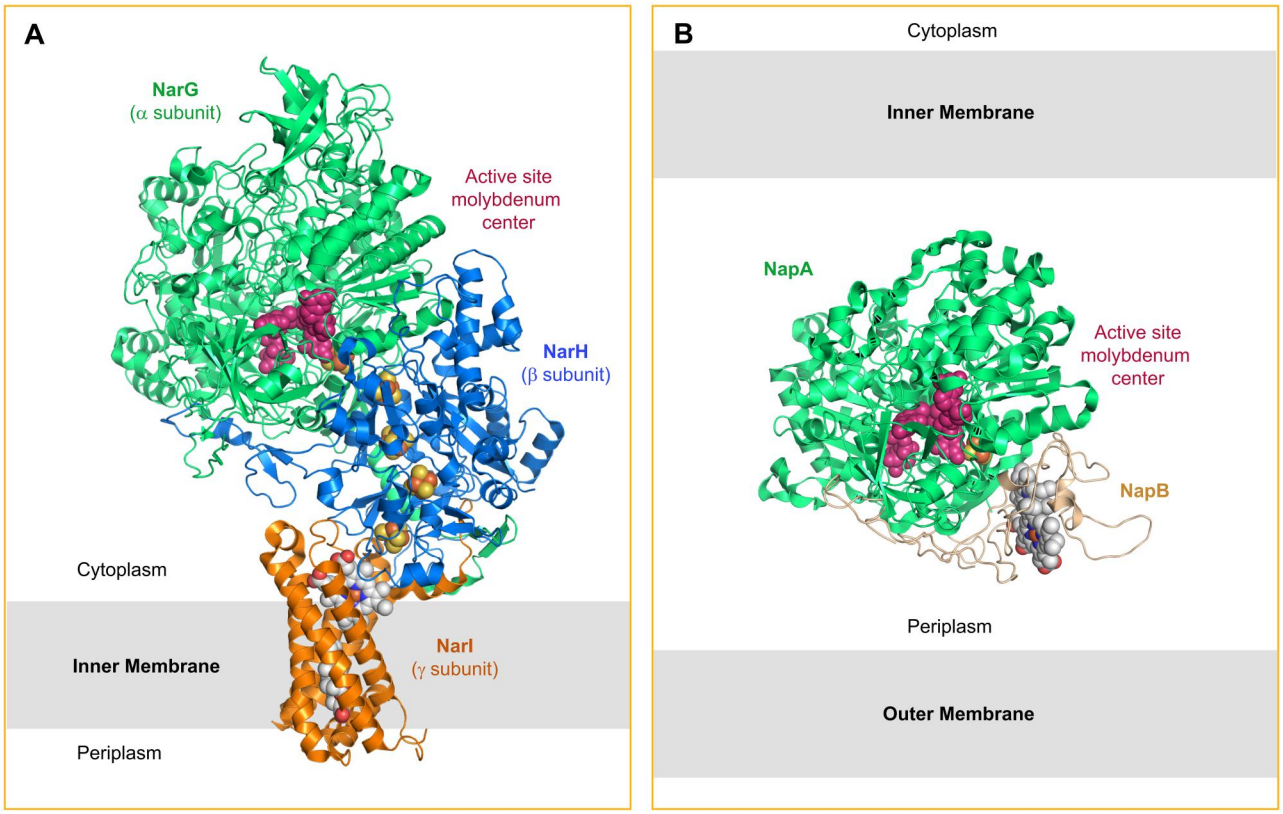


Figure 3. Representative structures of bacterial dissimilatory nitrate reductases that are members of the dimethylsulfoxide (DMSO) reductase family. These reductases include: (A) respiratory nitrate reductase. The structure of the membrane-bound respiratory NarGHI complex is shown in a cartoon representation (PDB entry 1Q16). For clarity, only one ($\alpha\beta\gamma$) unit of the $(\alpha\beta\gamma)_2$ dimer is shown. The α or G subunit (green) harbors the active site and one 4Fe/4S; the β or H subunit (blue) contains four 4Fe/4S; lastly, the membrane-spanning γ or I subunit (orange) contains two b-type hemes (grey). 4Fe/4S centers (orange/yellow) are shown in the protein structure, whereas the molybdenum-cofactor active site (purple) converts nitrate to nitrite. NarGHI operates in concert with transporters, such as NarK or NarT, that eliminate nitrite from a bacterium. (B) Periplasmic nitrate reductase. The structure of the NapAB complex localized in the periplasm is shown in a cartoon representation (PDB entry 1OGY). The A subunit (green) harbors the active site (purple) and one 4Fe/4S; the B subunit (tan) contains two c-type hemes (grey). Inner membrane associated NapC and/or NapGH (not shown) usually provide electrons to NapB, which are then transferred to the molybdenum-containing active site in NapA that facilitates the nitrate to nitrite reaction.

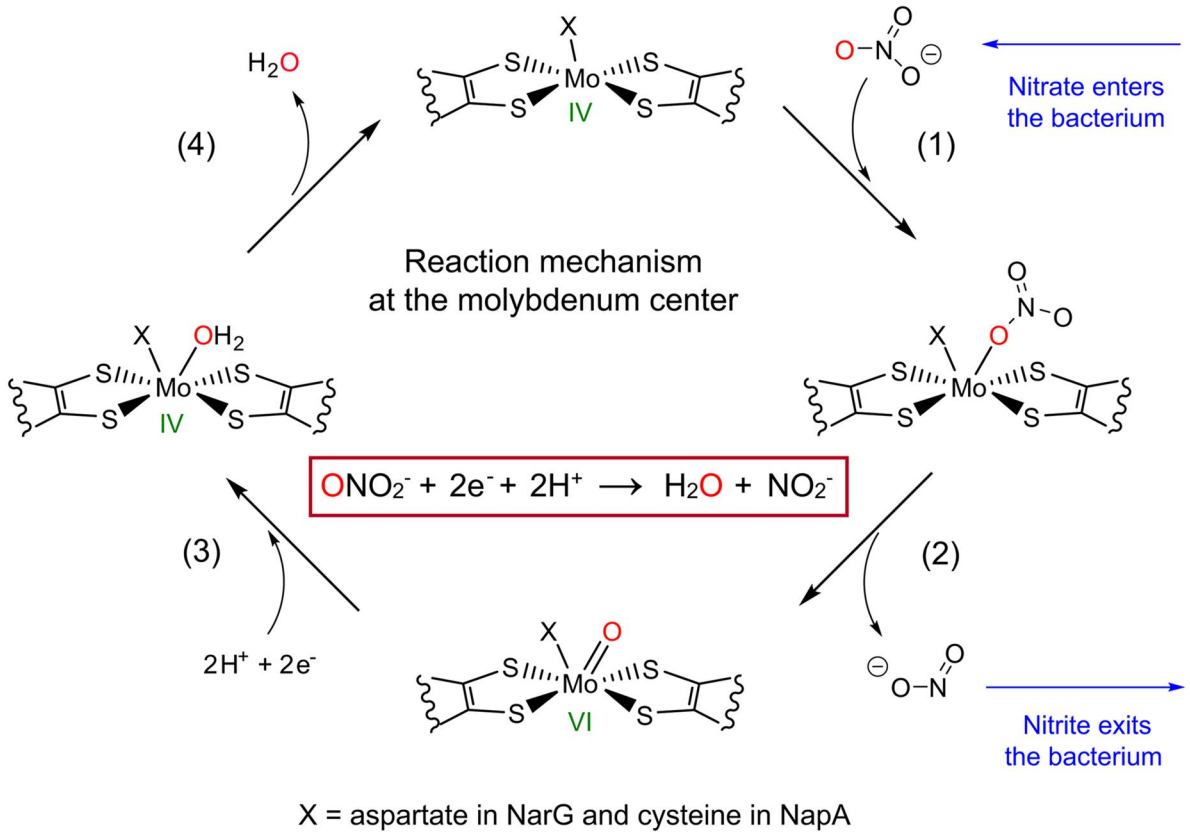


Figure 4. Nitrate reduction occurring at the molybdenum center in NarG and NapA subunits. Dissimilatory nitrate reductases, such as respiratory nitrate reductase that contains NarG and periplasmic nitrate reductase that contains NapA, are members of the molybdenum-containing dimethylsulfoxide (DMSO) reductase family. NarG usually contains an aspartate ligand attached to the molybdenum center, whereas NapA usually contains a cysteine. NarG and NapA are catalytic reductase subunits that facilitate a four-step oxotransferase mechanism which includes: Step 1, nitrate binds to molybdenum; step 2, a N–O bond of nitrate is cleaved and liberates a nitrite; step 3, two protons and two electrons are consumed and, lastly; step 4, a water molecule is released. The nitrite anion formed in step 2 can either be assimilated or released from the bacterium. The consumption of two protons from the cytoplasm during step 3 contributes to the overall transmembrane proton gradient utilized for bacterial ATP production. This enzymatic reaction is performed in the active site of respiratory nitrate reductases but also in periplasmic nitrate reductases, although the latter does not result in the production of ATP. These enzymes, if present and active, can contribute to extracellular nitrite accumulation.

Sapkota et al. 2010; Law et al. 2016; Tyx et al. 2016; Smyth et al. 2017; Rivera et al. 2020; Rivera and Tyx 2021).

Transcriptional regulation of the *nap* operon is complex and involves a number of regulatory proteins that sense environmental conditions, such as O_2, NO_3^-, carbon, iron, and molybdenum (Peters et al. 1987; Korner et al. 2003; Durand and Guillier 2021). Those last two elements are important for the biosynthesis of [4Fe/4S] centers and molybdenum cofactor biosynthesis (encoded in the *moaA* operon), respectively–both cofactors are essential components for catalytically competent NapA (Palmer et al. 1996; Vergnes et al. 2004). Periplasmic nitrate reductases can generate NO_2^- when NO_3^- is present with adequate O_2 and excess carbon, whereas some periplasmic nitrate reductases generate NO_2^- under low O_2 conditions. *Ralstonia* are betaproteobacteria and tobacco plant pathogens found in some ST products (Tyx et al. 2016; Rivera et al. 2020) that express *nap* genes under both normal and low O_2 levels, which may allow NO_2^- to be generated during various processing stages with varied O_2 status. *Ralstonia* can also contain *nar* genes (Sparacino-Watkins et al. 2014; Maia et al. 2017; Rivera et al. 2020).

Although molybdenum is rare in nature, it is found in a number of nitrogen-cycle enzymes. Indeed, among bacteria, at least 50 different molybdoenzymes have been reported (Mendel and Bittner 2006). As mentioned, a single molybdenum (Mo) atom is present in the active sites of enzymes that catalyze redox reactions involving carbon, sulfur, and nitrogen. Both dissimilatory nitrate reductases, respiratory nitrate reductases and periplasmic nitrate reductases are members of the dimethylsulfoxide (DMSO) reductase family that contains a single Mo atom held in place by four sulfur ligands in two pyranopterin units, and an amino acid residue, such as aspartate or cysteine in NarG and NapA, respectively. The multi-step nitrate reduction cycle is shown in Figure 4. In the oxotransferase reaction, Mo can form a single bond with one of the three oxygen atoms in a NO_3^- anion to yield a Mo–O–NO_2^- intermediate (step 1) that readily forms Mo═O while releasing NO_2^- (step 2) that can either be assimilated or exported out of the bacterial cell. To restore Mo═O back to Mo in the active site, two protons and two electrons are consumed (step 3), and a single water molecule is lost (step 4), which restores Mo so a subsequent nitrate reduction can occur (Maia et al. 2017). The active site of periplasmic nitrate reductase (NapA) catalyzes nitrate reduction in a manner similar to respiratory nitrate reductase (González et al. 2006, 2017; Sparacino-Watkins et al. 2014).

Certain bacterial species (e.g., *Bacillus, Enterococcus*) can generate and release nitrite under aerobic and anaerobic conditions (Wong and Flint 2019). Moreover, when O_2 is not available and

aerobic respiration is not occurring, respiratory nitrate reduction in certain species can provide an alternative means of producing ATP. As mentioned earlier, low O_2 conditions, such as present during fermentation, can trigger the expression of NarGHI proteins. When the NarGHI complex is assembled and operational, two protons are consumed in the cytoplasm during the nitrate reduction reaction in the NarG subunit (not shown) (Figure 4, step 3), and two protons are liberated into the periplasmic space due to oxidation of each QH_2 within the NarI subunit. This produces a net balance of $4H^+$ between the cytoplasm and periplasmic space for each nitrate reduced by NarGHI. The resulting transmembrane proton gradient harnessed by ATP synthase drives the production of ATP (Figure 5) (González et al. 2017).

Not all nitrite that is produced in bacteria is released as some bacteria contain NirBD or a similar nitrite reductase that reduce nitrite to ammonium that is then assimilated into nitrogen-containing biomolecules (Figure A1). Nitrite that is exported moves to the periplasm via NarK, NarT, NrtABC, or other ion transporters and then through porins (González

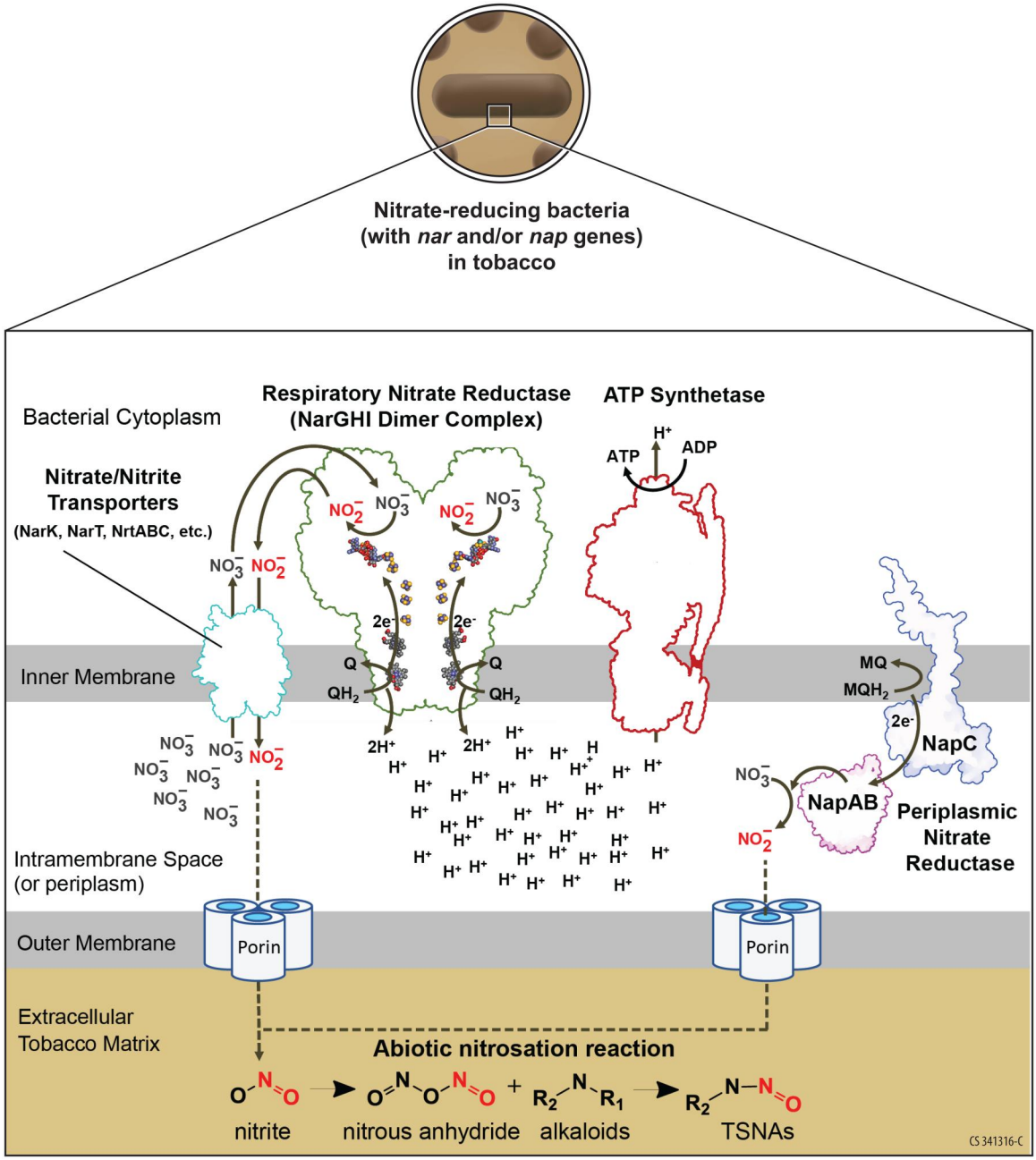
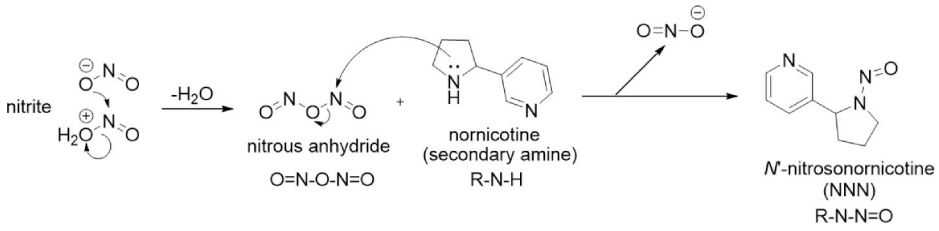


Figure 5. Potential routes of TSNA formation include bacterial nitrite production and release, followed by abiotic nitrosation. A variety of bacteria, present in smokeless tobacco products, can perform aerobic respiration utilizing O_2 as a terminal electron acceptor but can also contain respiratory nitrate reductases (NarGHI). Under low O_2 levels, bacteria expressing NarGHI use nitrate (NO_3^-) as an alternate electron acceptor and generate a transmembrane proton motive force that drives ATP production. Because nitrite can be toxic in certain bacteria, it is either assimilated or released into the extracellular environment. Assimilation of nitrite can occur if NirBD (not shown) or another nitrite reductase is present in the cytoplasm. Some bacteria may contain NapAB in the periplasm that can generate and release nitrite. NapC but also NapGH (not shown) can provide electrons required for NapAB to reduce nitrate to nitrite. When nitrite is released by bacterial porins into tobacco, it can react with tobacco alkaloids, such as nicotine and nornicotine, to form carcinogenic TSNAs via chemical nitrosation. Other nitrite-producing enzymes exist in other bacteria or fungi that may be present in tobacco. Nitrate-reducing bacteria can have different sizes and morphologies.



(1)

et al. 2017) to reach the tobacco matrix where nitrite can react with alkaloids to form TSNA (Figure 5). In bacteria that contain *nar* and *nir* genes, and when nitrite levels are high relative to nitrate, the *nar* operon that facilitates nitrite production/export is down-regulated, and *nir* genes that encode the nitrite reductase nirBD are up-regulated, thus favoring assimilation where nitrite is reduced to ammonium (NH_4^+) and then incorporation into nitrogen-containing biomolecules (Rabin and Stewart 1993; Noriega et al. 2010). Section 2.6 deals with chemical nitrosation of tobacco alkaloids via reactive NO_x gases released during curing or via nitrite released from certain microorganisms during processing.

2.6. *Abiotic* N-*nitrosation in the formation of* N-*nitrosamines*

Nitrosation of tobacco alkaloids can occur during flue-curing when NO_x gases, such as nitric oxide (NO) or nitrogen dioxide (NO_2) formed from nitrate or nitrite, react directly with tobacco alkaloids to form TSNA (Wang et al. 2017). NO_x gases, formed in the smoke from wood combustion that is present during fire-curing, can react with tobacco alkaloids to produce TSNAs. Fire-curing, which can also introduce carcinogenic PAHs, is used to make cigars and some ST products such as moist snuff or dry snuff (Di Giacomo et al. 2007; Ellington and Boyette 2013; Hearn et al. 2013; Lawler et al. 2013; Tobacco Guide 2023). Nitrosation can also occur when nitrite (NO_2^-), formed by bacterial dissimilatory nitrate reductases, is converted to nitrous anhydride (N_2O_3), which then reacts with tobacco alkaloids to form TSNAs (Figure 5). The nitrosation reaction of nitrite with tobacco alkaloids in forming TSNAs covered in this section has been studied more in the past than the bacterial formation of nitrite (Section 2.7). Research addressing nitrosation reactions of sodium nitrite ($NaNO_2$) with various secondary and tertiary alkaloids in aqueous solution provided an early understanding of the influence that pH, temperature, and substrate concentrations have on TSNA formation. Nornicotine, a secondary amine, is readily nitrosated by $NaNO_2$ to produce NNN, with maximum rates between pH 3.0 and 3.4 (Mirvish et al. 1977; Hecht et al. 1978).

The stability of nitrite that occurs as pH increases influences the reaction of nitrite with secondary and tertiary amines (Mirvish 1975). The nitrosation of nicotine to NNN occurs over a wide pH range (pH 2.0–7.0), while the nitrosation reaction of nitrite with nicotine produces lower NNK levels (Hecht et al. 1978). Under most conditions, the nitrosating agent of alkaloids is nitrous anhydride formed from the reaction of two molecules of nitrous acid (HONO) formed from nitrite. This reaction occurs most readily at pH 3.4 but has been observed over a wide range of pH values that may exist throughout tobacco product processing steps (Mirvish 1975; Mirvish et al. 1977). Besides forming TSNAs, nitrosation can also form volatile nitrosamines, nitrosamino acids, and other nitroso compounds (Spiegelhalder and Fischer 1991; Wiernik et al. 1995; Wahlberg et al. 1999; Rodgman and Perfetti 2013). Nitrite-initiated formation of the carcinogen NNN via the abiotic process of nitrosation is shown in the following equation:

2.7. *Final smokeless tobacco product chemistry*

As already shown in Section 2, tobacco becomes more chemically complex as it proceeds through production of the final ST product. Tobacco processing methods vary widely worldwide in terms of the different tobacco species/varieties used, types of curing and processing used, nicotine content, pH, and non-tobacco ingredients (both plant-based and chemicals). ST consists of manufactured and cottage-made products and custom, hand-made ST preparations (Hatsukami et al. 2014). Descriptions and chemical information of representative ST products made and used globally are published (Hatsukami et al. 2014). Worldwide, ST products are available in a wide variety of formats, including twisted tobacco leaves, loose tobacco, ground tobacco, moist tobacco, dry tobacco, charred tobacco, tars, pastes, dentifrice powders, chewing gums, pressed cake, pellets, pulverized tobacco, dissolvable tobacco, and mixtures of tobacco flakes with other materials (Hatsukami et al. 2014).

In the U.S., moist snuff is very popular, whereas snus products (some made in Sweden) are pasteurized snuff-type products, and are also popular (Rutqvist et al. 2011; Hatsukami et al. 2014; Lawler et al. 2020). In India, where ST product use is highest in the world, a wide array of products, including khaini, zarda, gul, gutkha, mishri, mawa, bajjar, gudhaku, tapkeer, and quiwam are common. Indeed, the variety of product types and brands, with distinct processing regimes and product ingredients, produced and used in India are very numerous. Tuibur does not contain any tobacco leaf material but it is made by bubbling tobacco smoke through water; this product is gargled or held in the mouth. Another unique product is creamy snuff, which is a tobacco-containing toothpaste (Stanfill et al. 2011; Hatsukami et al. 2014; Sinha et al. 2018). In addition, custom hand-made ST preparations, such as betel quid with tobacco (paan) or tombol are made by

combining tobacco or tobacco products with areca nut, spices, alkaline agents, and numerous other ingredients. Many of the products from India and surrounding countries are now marketed to migrant communities in Western countries, such as the U.K. and U.S. (Hatsukami et al. 2014).

Indeed, all of the factors and processes mentioned so far in Section 2 can contribute to the highly variable and complex chemical nature of tobacco (Rodgman and Perfetti 2013) observed among the spectrum of ST products, ranging from pasteurized snus products with very low TSNAs to fermented and aged products with very high TSNA levels (e.g. toombak and other types of snuff) (Idris et al. 1991, 1998; Di Giacomo et al. 2007; Richter et al. 2008; Stanfill et al. 2011; Lawler et al. 2013, 2020; Hatsukami et al. 2014). Regardless of how ST products are used orally, nasally or as a dental application, carcinogenic NNN and NNK and also other TSNAs can be absorbed (Tomar and Henningfield 1997; Hatsukami et al. 2014). Two other TSNAs, *N*′-nitrosoanabasine (NAB) and *N*′-nitrosoanatabine (NAT), are present in ST products but have weaker or no demonstrated carcinogenicity, respectively (Kroes and Kozianowski 2002; IARC 2007). NNN and NNK demonstrate remarkable carcinogenic activities typical of the structural class of *N*-nitrosamines. The carcinogenicity of this class of compounds is so potent that the *N*-nitrosamine group is excluded from the widely used "threshold of toxicological concern" approach to risk assessment as there is generally no entirely safe level of exposure to these compounds (Kroes and Kozianowski 2002).

The concentrations of NNN and NNK in ST products are far greater than those of any other potent carcinogen found in other products designed for human consumption (Hatsukami et al. 2014). The U.S. Food and Drug Administration (FDA) has proposed regulations of NNN in finished tobacco products at a level not exceeding 1 μg/g dry weight tobacco (Federal Register 2017). A recent survey of nine ST products purchased between 2008 and 2017 in the U.S. had levels ranging from 2 to 10 μg/g dry weight for NNN and 0.8–3.7 μg/g dry wt. for NNK. Year-to-year variations in NNN and NNK levels are due in part to diverse farming practices and processing technologies and variable weather patterns (Fisher et al. 2012; Oldham et al. 2020; Tobacco Guide 2023). Human carcinogens can be present at high concentrations in products in South Asian countries. High levels of NNN and NNK were observed in ST products sold in India. In one study, khaini contained higher levels of NNN (39.4–76.9 μg/g) and NNK (2.34–28.4 μg/g) than zarda products with lower levels of NNN (4.81–19.9 μg/g) and NNK (3.09–16.4 μg/g) (Stepanov et al. 2005). A study of ST products from Bangladesh found wide ranges of NNN (1.1–59 μg/g) and NNK (0.15–34 μg/g) (Nasrin et al. 2020). However, none of the products surveyed approach the extreme levels in toombak from Sudan (Idris et al. 1991).

In the U.S., moist snuff is very popular, whereas snus products (some made in Sweden) are pasteurized snuff-type products, and are also popular (Richter et al. 2008; Rutqvist et al. 2011; Hatsukami et al. 2014; Lawler et al. 2020). In India, where ST product use is highest in the world, a wide array of products, including khaini, zarda, gul, gutkha, mishri, mawa, bajjar, gudhaku, tapkeer, and quiwam are common. Indeed, the variety of product types and brands, with distinct processing regimes and product ingredients, produced and used in India are very numerous. One product called tuiber differs from all other ST products as it does not contain any tobacco material but it is made by bubbling tobacco smoke through water; this product is gargled or held in the mouth (Stanfill et al. 2011; Hatsukami et al. 2014). In addition, custom handmade ST preparations, such as betel quid with tobacco (paan) or tombol are made by combining tobacco or tobacco products with areca nut, spices, alkaline agents, and numerous other ingredients. Many of the products from India and surrounding countries are now marketed to migrant communities in Western countries, such as the U.K. and U.S. Due to non-standardization and heterogeneity in ingredients, formulations, and packaging, the same product brand purchased at different times or locations may vary widely in their chemical content (Hatsukami et al. 2014).

3. Exposure to *N*-nitrosamines and other carcinogens due to smokeless tobacco use

3.1. Smokeless tobacco usage and the human body

Regardless of how TSNAs are formed in ST products, human exposure occurs when TSNAs are absorbed when a portion or "dip" of an ST product or preparation is inserted in the oral cavity, then chewed, sucked, or held in contact with the buccal membranes. Some products consist of small teabag-like pouches that contain a pre-portioned amount of tobacco (moist snuff, snus, and khaini) that is placed inside of the mouth (inside of the cheek or under the upper or lower lip); absorption of nicotine and other chemicals occurs during use (Tomar and Henningfield 1997; Konstantinou et al. 2018). Loose tobacco or pouches may be held in the mouth, chewed, or sucked for a short (∼30 min) or an extended period of time (multiple hours) to obtain nicotine (Mehrotra et al. 2020). Products such as tobacco-containing tooth powder or toothpaste may be used to brush the teeth or applied as a dentifrice. Some ST products that are dry powders, such as tapkeer, taaba, tawa, toombak, dry snuff, etc. are sniffed nasally (Hatsukami et al. 2014).

Nicotine, well known for its addictiveness, also increases the number of nicotinic acetylcholine receptors (nAChRs) that are expressed in cells. Because NNK and NNN are structurally similar to nicotine, those two TSNA molecules can also bind to nAChRs and may promote cancer cell proliferation by creating a microenvironment for tumor growth (Xue et al. 2014; Locker et al. 2016). Nicotine is also suspected to contribute to cancer promotion and progression by activating nAChRs; for example, in stomach cancer, both nicotine and NNK enhance cell proliferation through nAChR and other receptors (Dang et al. 2016).

3.2. Absorption, metabolism, and elimination

Because different tobacco products contain a wide range of TSNA concentrations, human exposure also varies from low to high levels (Hatsukami et al. 2014). Once absorbed, TSNAs enter the bloodstream and are then transformed by

human metabolic pathways such as generation of TSNA glucuronides that are excreted or metabolically activated to diazohydroxides and intermediates that attack nucleophilic centers in the cell, causing DNA adduct formation (Figure 6). If DNA adducts are not repaired or are incorrectly repaired, this can result in mutations in certain oncogenes (e.g. RAS) or tumor suppressor genes (e.g. p53) that result in altered protein function, uncontrolled cell proliferation, and, in many cases, cancer (Ma et al. 2019). Mortality rates can be high, especially among people who use ST products with high TSNA levels and/or lack adequate medical intervention (Idris et al. 1998).

The amount of TSNAs and other ST-related compounds absorbed by the body depends on the amount of products used; product TSNA concentration; frequency of use (portions/day); intensity of product chewing or sucking; time duration that ST product remains in the oral cavity and in contact with oral tissue; moisture content of product; oral pH; and salivary volume (Lemmonds et al. 2005; Hatsukami et al. 2014; Xia et al. 2021). During ST product use, TSNAs are absorbed across oral membranes and enter the human body where they are metabolized, leading to the formation of the carcinogenic NNK-metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and also NNAL-glucuronide and NNN-glucuronide. NNAL is generally excreted in urine with a relatively long half-life of approximately 3 weeks; for that reason, urinary NNAL is a sensitive and specific biomarker for NNK exposure and useful as a surrogate biomarker of exposure to all TSNAs in ST products (Xia et al. 2021).

Reverse dosimetry has been used to estimate the internal dose of carcinogenic NNK based on the NNK metabolite NNAL in urine in people who used ST products made in the U.S. (Wei et al. 2016). Similar dose calculations can be made for NNN based on urinary NNN concentrations, although urinary NNN measurement presents unique challenges (Lagerwerf et al. 1998; Stepanov et al. 2005). In one study, maximum salivary levels of NNN in people who chewed toombak were eight times higher than in people who chewed tobacco; salivary NNK in those who chewed toombak was more than 30 times higher than in those who chewed tobacco (Idris et al. 1992).

Important human metabolic pathways for NNK, NNN, and NNAL are illustrated in Figure 6. When NNK enters cells in human tissues, including the oral mucosa, it is metabolized to NNAL by enzymes of the short chain dehydrogenases/reductases (SDRs) superfamily and the aldo/keto reductases (AKRs) superfamily (Stapelfeld et al. 2017). Even with that structural change, NNAL still has carcinogenic activity similar to NNK. TSNAs that are glucuronidated are more water soluble, less toxic, and are readily excreted. Glucuronidation of NNAL, catalyzed mainly by the UDP-glucuronosyltransferase (UGT) isozymes 2B10 and 1A17, is a detoxification reaction. Glucuronide conjugation of NNAL occurs at either the hydroxyl group or the pyridine nitrogen, prior to excretion (Hecht 1998; Balbo et al. 2014; Hecht et al. 2016; Kozlovich et al. 2019). NNN can undergo glucuronidation by UGTs at the pyridine nitrogen, resulting in NNN-glucuronide that is excreted (Stepanov and Hecht 2005).

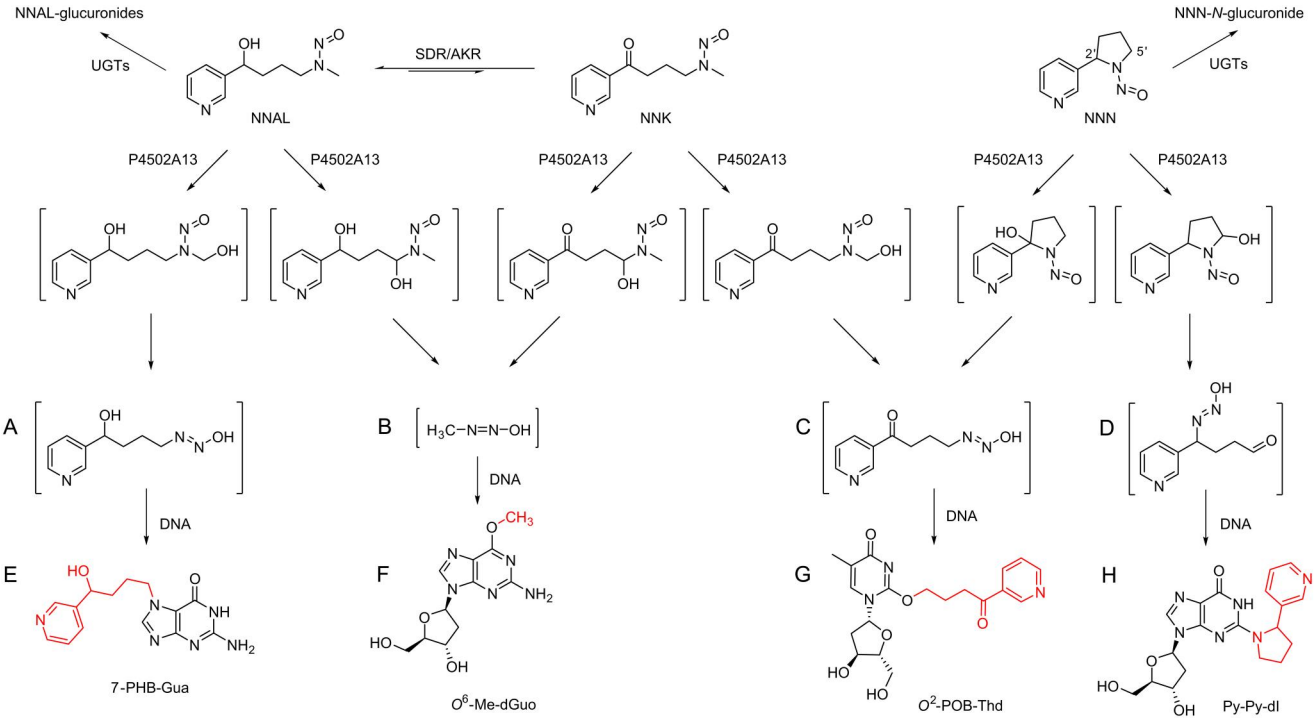


Figure 6. Human absorption and metabolism of NNAL, NNK, and NNN to reactive diazohydroxides and DNA adducts. Nitrosamines (R_2–N–N=O) enter somatic cells where they are metabolized to diazohydroxide, R–N=N–OH, and transient intermediates that can react with DNA to form adducts that can contribute to deleterious nucleotide base changes, ultimately leading to various cancers. Intermediates are shown in brackets. The metabolism of NNAL, NNK, and NNN by the cytochrome P450 isozymes 2A13 and other P450 enzymes lead to reactive intermediates including: (A) pyridylhydroxybutyl (PHB) diazohydroxides, (B) methyl diazohydroxides, (C) pyridyloxobutyl (POB) diazohydroxides, and (D) aldehydic diazohydroxides that can form various DNA adducts, shown in red. Four representative DNA adducts that are formed include: (E) 7-(pyridylhydroxybutyl)guanine, (F) O^6-methyldeoxyguanosine, (G) O^2-(pyridyloxobutyl)thymidine, and (H) 2-(2-(3-pyridyl)-*N*-(pyrrolidinyl)-2′-deoxyinosine. Numerous other adducts can form from exposure to these diazohydroxides.

3.3. Adduct formation

TSNAs can be metabolized and excreted, or converted to reactive intermediates that form DNA adducts (Figure 6). In order to exert their carcinogenic effects, all nitrosamines require hydroxylation of a carbon atom adjacent to the *N*-nitroso group (–N–N═O) in a process called α-hydroxylation (Preussmann and Stewart 1984). Essential metabolic transformations in ST users are catalyzed by cytochrome P450 (CYP450) enzymes that function as monooxygenases with a heme cofactor. For NNK, NNAL, and NNN, α-hydroxylation catalysis occurs most efficiently when facilitated by the CYP450 isozymes 2A13 and 2A6 (Jalas et al. 2005; Wong et al. 2005), which generate α-hydroxy compounds that are unstable and spontaneously decompose to aldehydes and diazohydroxides.

TSNAs (R_2–N–N═O) that are not glucuronidated and then eliminated can be further metabolized to diazohydroxides (R–N═NOH) and transient intermediates, including diazonium ions (R–N≡N^+) and carbocations (R–CH_2^+) (Figure 6). These intermediates are electrophilic and readily react with nucleophilic sites in human DNA, RNA, protein, and also with water to generate alcohol-containing metabolites. Reactions of diazonium ions and carbocations with nucleophilic sites in DNA are considered crucial to chemical carcinogenesis as various types of DNA adducts can lead to mutations (Ma et al. 2019). Examples of DNA adducts formed from NNAL, NNK, and NNN include 7-PHB-Gua, O^6-Me-dGuo, O^2-POB-Thd, and Py-Py-dI illustrated in Figure 6; other adducts are also possible. Only one structural representative of the four adduct types is illustrated, but each pathway shown can result in formation of multiple DNA adducts. Arecoline from areca nut likewise forms areca-specific nitrosamines and a number of reactive aldehydes that in turn generate several reactive diazohydroxide intermediates (Franke et al. 2015).

Biomarker studies have investigated the metabolism of NNK and NNN in ST users (Hecht et al. 2016). Human metabolic pathways either detoxify, primarily via glucuronidation, or activate NNN, NNK, or NNAL to diazohydroxides and related intermediates (Figure 6). Evidence consistent with the 2′-hydroxylation pathway of NNN and/or the methyl hydroxylation of NNK has been obtained. Due to a chiral center at the 2′ carbon position, (*S*)-NNN and (*R*)-NNN are essentially molecular mirror images; however, of these two enantiomers, (*S*)-NNN causes more total adduct formation than (*R*)-NNN (Zhao et al. 2013). The 5′-hydroxylation of (*S*)-NNN predominates in human enzyme systems, forming the py-py-dI adduct in greater amounts than POB adducts formed by 2′-hydroxylation (Zarth et al. 2016). Hydroxylation of NNK can occur at the α-methyl and α-methylene carbon positions, forming keto alcohol, keto aldehyde, and two diazohydroxides capable of producing pyridyloxobutylating or methylating agents that form POB-DNA or methyl-DNA adducts, respectively (Figure 6) (Hecht et al. 2016).

If POB-DNA adducts and related adducts remain unrepaired by either evading or overwhelming healthy cellular repair systems, the result can be miscoding leading to DNA mutations in somatic cells. Besides reacting with human DNA, POB species react to form hemoglobin adducts that can be quantified using the mass spectrometric measurement of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) released by hydrolysis. This analytical approach is a minimally-invasive means (i.e., typical blood draw) of accessing POB exposure (Hecht et al. 2016). If these mutations occur in crucial regions of specific genes, such as the *RAS* oncogenes or the p53 tumor suppressor gene, the result can be the loss of healthy cellular growth control, and, ultimately, the development of cancer (Warnakulasuriya and Ralhan 2007). This process of adducts leading to mutations is perhaps most clearly illustrated for O^6-Me-dGuo, which is formed by methylene hydroxylation of NNAL or NNK. The persistence of O^6-Me-dGuo in specific rat tissues, where tumors developed following treatment with *N*-methyl-*N*-nitrosourea, supported the hypothesis that the formation of O^6-Me-dGuo caused miscoding in DNA (Margison and Kleihues 1975). Elegant experiments demonstrated unequivocally that O^6-MeG causes G-to-A mutations in DNA (Loechler et al. 1984).

Rats treated with a dose of 5 ppm NNK in the drinking water for 70 weeks had a high incidence of lung tumors; the main rat target tissue of NNK is the lungs (Hecht 1998). The major DNA adduct was O^2-POB-dThd, followed by 7-POB-Gua, with much lower amounts of O^6-POB-dGuo and O^6-Me-Gua; levels of all DNA adducts decreased over the 70 week period (Balbo et al. 2013). Treatment of rats with (*S*)-NNN is highly tumorigenic, especially in the esophagus and the oral cavity. It is possible that inefficient repair of 7-POB-dGuo may be one reason for carcinogenesis related to NNN exposure (Zhao et al. 2013). For NNN in ST products, the predominant enantiomer is (*S*)-NNN. A 14-ppm dose of (*S*)-NNN in drinking water administered to 20 male F-344 rats for 17 months resulted in 89 benign and malignant tumors in the oral cavity. Of the rats treated with (*S*)-NNN, 100% developed tumors, with a significant number of esophageal tumors; this treatment also impacted tissues in the head and neck, such as the buccal mucosa, gingival mucosa, tongue, soft palate, epiglottis, and pharynx. Treatment of rats with (*R*)-NNN alone was not active in forming tumors, but a mixture of (*S*)-NNN and (*R*)-NNN together produced more tumors than treatment with (*S*)-NNN alone. This finding suggests that (*R*)-NNN may act as a co-carcinogen enhancing the tumorigenic effects of (*S*)-NNN (Balbo et al. (2013).

In a different study, a mixture of NNN and NNK swabbed inside the oral cavity and on the lips of rats induced a significant incidence of oral cavity tumors (Hecht et al. 1986), most likely due to NNN; because when the NNK dose is high in the drinking water, the main rat target tissue is the lungs (Hecht 1998). A 5-ppm dose of NNK in the drinking water administered for 70 weeks induced lung tumors in 100% of the treated rats (Balbo et al. 2014). Nasal tumors are commonly observed in rats treated with NNK by injection (Hecht 1998). While one of the main carcinogenic effects of ST use in humans is induction of oral cavity cancer, one recent epidemiologic study demonstrated a significant incidence of lung cancer among people who used ST but not combustible tobacco. This is consistent with NNK being a powerful lung carcinogen, even when the absorption occurs orally (Andreotti et al. 2017).

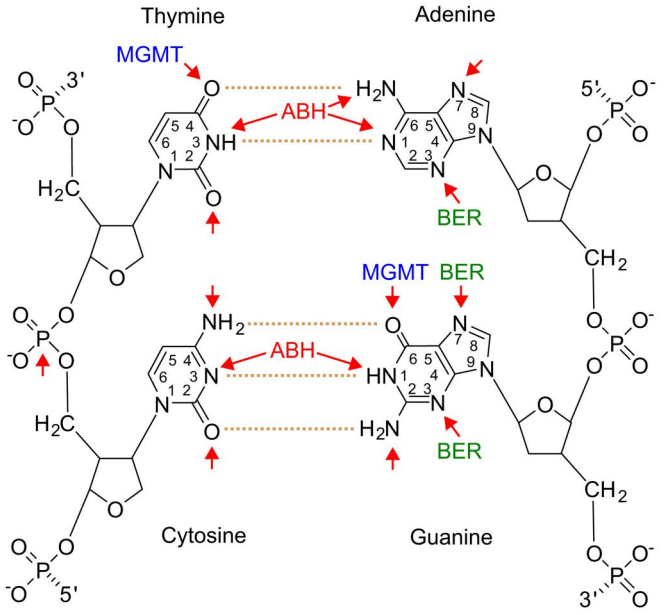


Figure 7. Smokeless tobacco constituents cause adducts that result in DNA damage at all four nucleotide bases that generally require repair to prevent mutations. All of the red arrows show DNA alkylation attack sites due to exposure to *N*-nitrosamine and another common nitrosamine, *N*-nitrosodimethylamine (NDMA). Adducts can form at oxygens and nitrogens throughout the nucleotide bases, except the ring nitrogen in the *N*-glycosidic bond (i.e. N9 in purines; N1 in pyrimidines). Red arrows with labels show the site of adduct formation and their mechanism of repair if known, including O^6-alkylguanine-DNA alkyltransferase (MGMT), alkB homologous protein 2 (ABH), and base excision repair (BER). Red arrows without labels represent adduct sites that are either not repaired (e.g. phosphotriester) or the repair mechanism is not presently known. TSNA (NNN, NNK, and NNAL) forms alkyl adducts that damage DNA at adenine (N1, N3, and N^6 positions), cytosine (O^2 and N^4), guanine (O^6, N^2, N3, and N7), and thymine (O^2 and O^4); and also on the phosphodiester backbone (Li et al. 2019; Ma et al. 2019). If areca nut is also present in a tobacco-containing product, arecoline can form adducts at the O^6 position on guanine and O^4 position on thymine (Lee et al. 2013). Some adducts, which are so damaging that a stretch of as many as 25–30 nucleotides must be removed by nucleotide excision repair, include those due to: metabolites of AFB_1 that form bulky adducts that attack the N7 position of guanine and PAH metabolites that attack the N^2 position on guanine (Szalat et al. 2018) (not shown).

In addition to TSNAs, BaP, which is a PAH compound and an IARC Group 1 carcinogen (Hatsukami et al. 2014), accumulates in fire-cured tobacco used to make moist snuff and dry snuff (Davis and Nielsen 1999; Tobacco Guide 2023). Human metabolism converts BaP to a reactive metabolite that reacts to form an *anti*-benzo[*a*]pyrene-7,8-diol-9,10-oxide-DNA adduct, a guanine adduct occurring at CpG mutation hotspots present at several codons in the p53 sequence (Barta et al. 2020).

3.4. Repair of DNA adducts

DNA adducts can originate not only from endogenous reactive species generated by human metabolic processes, but also from exogenous agents from environmental (air/water pollution, pesticides), occupational, or residential sources (diet, consumer products, medical drugs, azo dyes, and tobacco smoke) (Jackson and Bartek 2009). An important source of additional DNA adducts are carcinogens absorbed during the usage of ST products or other tobacco products (Hecht and Hatsukami 2022; Li and Hecht 2022). Complete repair of DNA adducts is crucial to genomic stability, genetic integrity of key genes, such as oncogenes and tumor suppressors, and the prevention of cancer. Several repair systems exist that detect adducts, remove them or revert the nucleotide bases back to their correct identity to prevent the formation of adduct-induced cancers (Hoeijmakers 2001; Xu-Welliver and Pegg 2002).

TSNAs, such as NNN, NNK, and NNAL, are found at some level in almost all ST products. These chemicals can form alkyl adducts that damage DNA at various ring positions on all four DNA nucleotide bases (Christmann and Kaina 2012; Li et al. 2019; Ma et al. 2019). Indeed, adducts attack almost all nitrogens and oxygens in the nucleotide ring structures, yet some of those sites are either not repaired or a repair mechanism is not presently known (Figure 7). It should be noted that NNAL and NNK form carbonium ions that are S_N1 alkylating agents, which directly react with biological molecules with high potency for alkylating oxygen moieties on DNA bases. Arecoline present in fermented tobacco can also form O^6-methylguanine and O^4-methylthymine (Lee et al. 2013). These two methylated bases are mispairing DNA lesions leading to point mutations. O^4-methylthymidine causes a very minor lesion; however, O^6-methylguanine represents about 6–8% of the total DNA methylation products (Beranek 1990). Both lesions are repaired by MGMT (discussed in detail next), which plays a key role in maintaining genome stability and cancer prevention (Margison and Santibáñez-Koref 2002; Kaina et al. 2007; Pegg 2011). DNA repair mechanisms shown in Figure 8 range from damage reversal by single enzymes such as O^6-methylguanine-DNA methyltransferase (MGMT, also designated as O^6-alkylguanine-DNA alkyltransferase) or alkB homologous protein 2 (ALKBH2)) to complex pathways, such as base excision repair (BER), nucleotide excision repair (NER), and DNA mismatch repair (MMR) that act to identify, remove, and repair alkyl damage caused by ST product constituents (Sharma et al. 2009). MGMT is a relatively small protein that transfers alkyl groups from the O^6-position of guanine or the O^4-position of thymine to its own active site (Figure 9). This action restores guanine or thymine in the DNA, but irreversibly inactivates MGMT–hence its designation as a *suicide enzyme*. MGMT preferentially removes methyl groups from the O^6-position of guanine, but can also remove with lower efficiency ethyl, propyl, butyl, benzyl and even larger pyridyloxobutyl (POB) groups, as shown in Figure 8(A) (Pauly et al. 2002; Lamb et al. 2014). MGMT naturally contains a zinc ion that is coordinated by four residues (C5, C24, H29, and H85), although zinc binding is not required for activity. Upon repair, MGMT carrying the alkyl group is tagged with ubiquitin and degraded by the 26S proteasomal pathway. When additional MGMT is needed, new protein is synthesized, which requires some time to replace and replenish the pool of functional MGMT (Sharma et al. 2009).

When confronting large numbers of unrepaired DNA adducts (Idris et al. 1991, 1992), the pool of functional MGMT enzymes may, in some cases, be inadequate to remove all of the adducts at the O^6-position of guanine or the O^4-position of thymine. If the number of adducts exceeds the number of MGMT molecules available at a given time in a certain cell that undergoes proliferation, this can lead to the formation

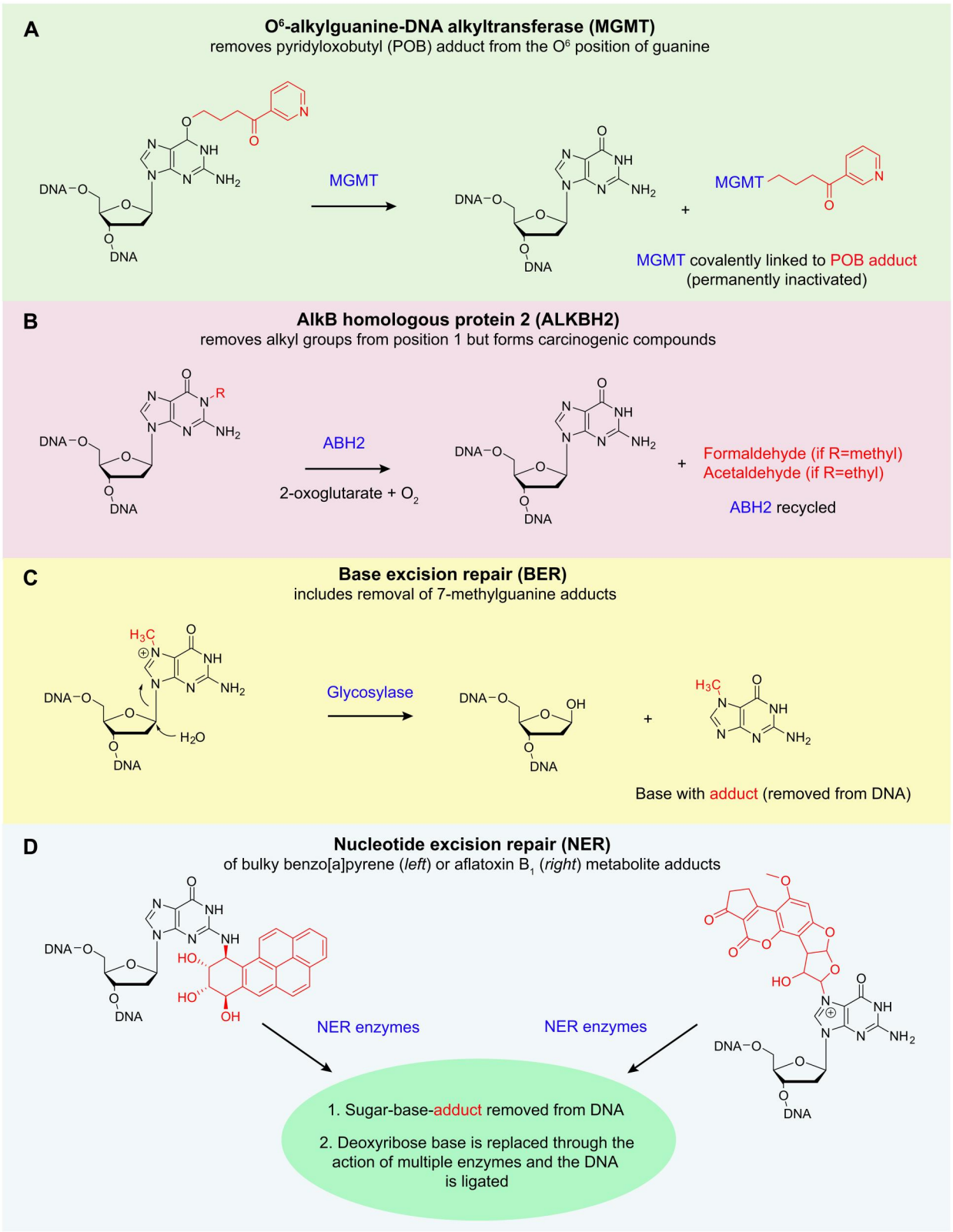


Figure 8. Overview of different repair mechanisms for guanine adducts. (A) Repair mediated by O^6-alkylguanine-DNA alkyltransferase (MGMT), (B) repair mediated by alkB homologous protein 2 (ALKBH2), (C) base excision repair, and (D) nucleotide excision repair.

of mutations. Indeed, ST consumption represents a chronic exposure of carcinogens to the proliferating mouth and esophagus epithelium, which likely results in MGMT tissue sequestration due to permanent adduct formation that may drive the process of genomic changes in ST users (Rohatgi et al. 2005; Sawhney et al. 2007; Lamb et al. 2014). Furthermore, MGMT expended in the repair of adducts caused by TSNAs and other ST-related compounds are not available to remove adducts formed from other endogenous and exogenous exposures (Sharma et al. 2009). The MGMT

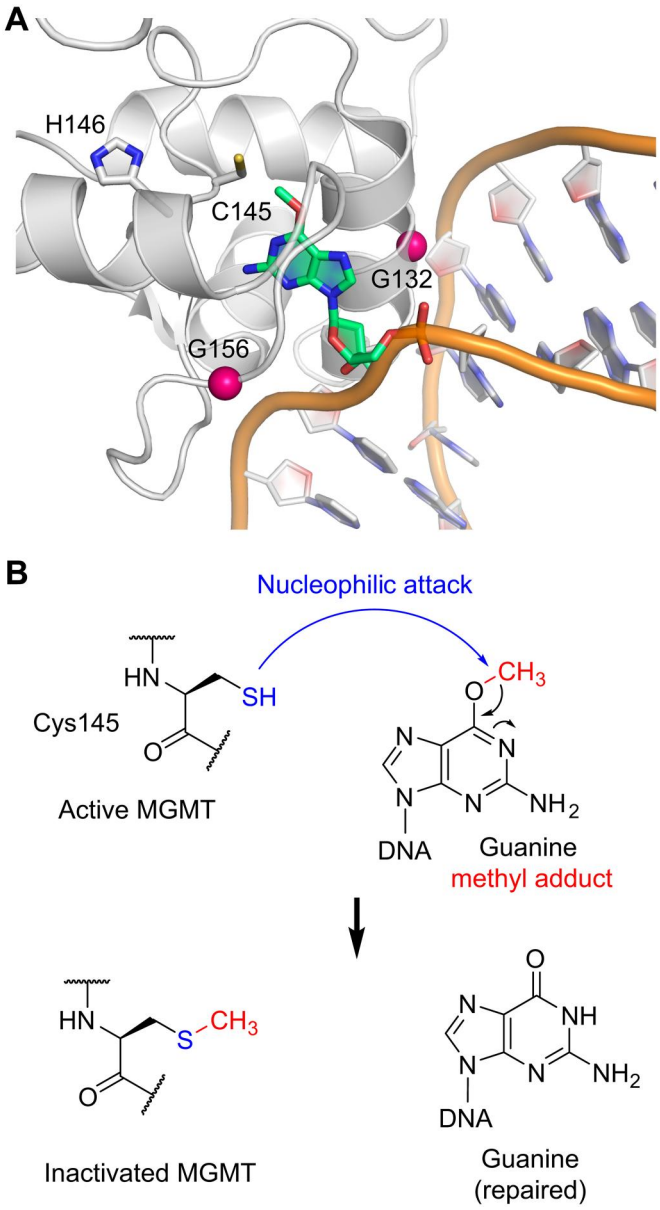


Figure 9. Action of human O^6-alkylguanine-DNA alkyltransferase (MGMT), a "suicide" enzyme, removes a single adduct caused by TSNAs and areca nut that is attached to the O^6-position of guanine or O^4-position of thymine. In this "suicide reaction", MGMT, which reverts modified DNA back to its natural state, is irreversibly inactivated so it must be eliminated and replaced. (A) Active site of human MGMT in complex with methylated DNA (PDB entry 1T38; Daniels et al. 2004). Certain TSNAs can form methyl adducts. The methylated guanine base is highlighted in green. Red circles indicate glycine residues mutated in cancer. MGMT functions by binding to a damaged section of DNA, rotating the aberrant nucleotide base out of the helix, and transferring a single alkyl group to the catalytic residue (C145) of MGMT, which becomes inactivated. The binding of an alkyl group to MGMT triggers a conformational change that signals ubiquitin to bind to an available lysine residue on the surface of the inactivated MGMT-adduct complex that tags it for proteolytic degradation. A histidine (H146) assists in catalysis via a structural water molecule. The crystal structure of this complex was determined with an inactive variant where the catalytic cysteine had been mutated to a serine but it has been modeled with the wild-type cysteine residue for the purpose of this figure. (B) The mechanism of methyl transfer from O^6-alkylguanine-DNA to the electronegative sulfur atom of the catalytic cysteine (C145) in the active site of MGMT.

activity in individuals is highly variable (Fagerberg et al. 2014). One possible outcome for people with low MGMT expression would be predisposition to cancer development following chronic consumption of ST products (Sharma et al. 2009). The tissue expression of MGMT is regulated through various transcription factors and epigenetic mechanisms (Christmann et al. 2011). Thus, if hypermethylation of CpG islands (the addition of methyl groups at the C5 position of cytosine in adjacent CG nucleotides) occurs in the promoter region of the MGMT gene, the gene is not expressed anymore and cells lack MGMT (*epigenetic silencing*).

Transcriptional silencing of MGMT by promoter hypermethylation has been observed in many tumors, with a frequency varying depending on tumor type: 56% in head and neck carcinoma; 8–50% in non-small cell lung cancer; 34–45% in glioblastoma; 28% in carcinoma; 24% in lung tumors; and 11% in pancreatic carcinoma (Sharma et al. 2009; Christmann and Kaina 2019). In workers exposed to *N*-nitrosamines, their DNA has a greater prevalence of O^6-methylguanine adducts. Reduction in MGMT activity or gene silencing through promoter methylation of the *MGMT* gene is associated with frequent mutations in *K-Ras* and *TP53* genes (Niture et al. 2007). MGMT expression in human tissues is highly variable (Margison et al. 2003) and shows, in human lymphocytes, remarkable inter-individual variability (Janssen et al. 2001). The varying levels of expression may make some tissues more susceptible to damage than other tissues due to persistence of adducts (Fagerberg et al. 2014; Jackson et al. 2019). The above studies demonstrate the importance and physiological relevance of DNA adducts and the vital importance of expression and proper functioning of the MGMT protein in removing adducts. Decreased gene expression of MGMT is associated with ST consumption in patients with oral precancerous lesions (Sawhney et al. 2007). In patients with oral squamous cell carcinomas, there was a significant association between loss of MGMT expression and poor health prognosis (Sawhney et al. 2007). An oral leukoplakia cell line treated with extracts of khaini, a fermented ST product used in India, exhibited a complete loss of MGMT expression (Rohatgi et al. 2005). Adducts at other ring positions on nucleotides that are not repaired by MGMT can possibly by repaired by ALKBH2, BER, or NER mechanisms (Sharma et al. 2009) (Figure 8).

Another direct DNA-damage reversal mechanism is mediated by ALKBH2 (Figure 8(B)). This protein is an α-ketoglutarate-dependent dioxygenase that acts as a single enzyme in repairing 1-methyladenine, 3-methylcytosine, 1-methylguanine, and other adducts in a one-step reaction. Unlike MGMT that is completely deactivated when repairing a single DNA base, the ALKBH2 enzyme active site is recycled to catalyze subsequent reactions. In order to function, ALKBH2 requires iron, molecular oxygen, and 2-oxoglutarate. Molecular oxygen and 2-oxoglutarate react with a methylated nucleobase to yield a completely repaired nucleobase, but also produces formaldehyde, a known human carcinogen produced in cellular metabolism. The amount of formaldehyde produced during the repair reaction is very likely so low that it has no adverse effects. However, if produced in high levels, it may pose a significant threat to genome stability. Formaldehyde (CH_2═O) can react to form DNA–protein crosslinks (DPCs)–essentially, a methylene bridge (–CH_2–) between a nucleophilic side chain of a lysine or cysteine on the surface of proteins with a DNA base, often at the C8 ring position on

guanine. If DPCs remain unrepaired, bulky DPCs are expected to interfere with DNA replication and transcription (Madison et al. 2012). Small DPCs can be removed by the NER mechanism, whereas larger DPCs generally have to be repaired by DPC proteases that digest the protein portion of a DPC until only a peptide adduct remains (Madison et al. 2012; Stingele and Jentsch 2015; Li et al. 2019; Ruggiano and Ramadan 2021). If ALKBH2 repairs an ethylated base, it forms acetaldehyde (CH_3CHO), which is a toxicant, mutagen and carcinogen (Seitz and Homann 2007). Formaldehyde and acetaldehyde that result also from other metabolic processes (Reingruber and Pontel 2018) can also be present in ST products that are fire-cured (Stepanov et al. 2008).

The main methylation products induced by activated NNAL and NNK are 7-methylguanine, 3-methyladenine, and O^6-methylguanine. 7-methylguanine and 3-methyladenine are not mispairing but result in apurinic sites due to spontaneous hydrolysis or repair intermediates, blocking DNA replication, which may result in DNA double-strand breaks and chromosomal aberrations (Kaina 2004; Ensminger et al. 2014). Chromosomal changes may lead to loss of tumor suppressor genes and thus become drivers of carcinogenesis. Experimentally, knockout mice that lack BER activity and are unable to repair some *N*-alkylation events are predisposed to cancer formation after treatment with azoxymethane, a methylating agent that induces DNA lesions similar to NNK (Wirtz et al. 2010). This supports the notion that BER activity may protect against cancer formation. Most of the *N*-methylpurines (i.e. methylguanine and methyladenine) are repaired by BER, which is a complex repair pathway involving a damage-specific DNA glycosylase, apurinic/apyrimidinic (AP) endonuclease, polymerase β, PARP-1, ligase III, and XRCC1 (Christmann et al. 2003) (see Abbreviations). For BER, the first step is executed by *N*-methylpurine-DNA glycosylase (MPG), which specifically recognizes the main adduct 7-methylguanine and other methylpurines produced in minor amounts (Figure 7(C)). Once the errant nucleotide is removed, AP endonuclease creates a single strand nick in the DNA helix on the AP site, and subsequent repair proceeds either by *short patch repair*, i.e. the replacement of a single nucleotide, or *long patch repair*, i.e. the replacement of up to 10 nucleotides. The short patch repair pathway involves the activity of a lyase and a polymerase, whereas the long patch repair pathway requires the action of a polymerase, a flap endonuclease, and a ligase (Christmann et al. 2003). The protective role of BER against tobacco-related carcinogens is not yet clear and needs to be investigated further.

Larger DNA adducts, such as those induced by tobacco-related carcinogens, are not repaired by BER but are repaired by NER (Szalat et al. 2018). BaP deposited on tobacco during fire-curing (Hearn et al. 2013) is absorbed during ST use and then converted to a metabolite that forms an adduct at the N^2 position of guanine (Figure 8(D)). NER is a critical repair mechanism that identifies and eliminates DNA crosslinks, larger DNA adducts formed from BaP and AFB1 (Szalat et al. 2018), and POB-related damage caused by NNK (Brown et al. 2008, 2009; Li et al. 2009). AFB1, formed by certain *Aspergillus* fungi, is one of the most toxic, hepatotoxic, mutagenic, teratogenic, and carcinogenic agents known (Deng et al. 2018) and is continually monitored in snus products produced under the Gothiatek Standard (Rutqvist et al. 2011). Aflatoxins can be produced by various fungal species (e.g. *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*) (Kumar et al. 2016; Rivera and Tyx 2021). AFB1, an IARC Group 1 carcinogen that has been found at very low levels in dry snuff and chewing tobacco, could form in products made in hot and humid climates (Zitomer et al. 2015). When absorbed by the human body, AFB1 is metabolically activated by liver CYP450 enzymes to a highly reactive and electrophilic epoxide, AFB1 exo-8,9-epoxide, that reacts to form the predominant adduct AFB1-N^7-guanine and other adducts (Wilson et al. 2016). The bulky AFB1-N^7-guanine adduct (Figure 8(D)) and other AFB1 adducts can cause a conformational change in chromatin and can block transcription. They are recognized by NER proteins, such as XPA, CSB, and CSA (abbreviations, see Appendix A1). NER is the key repair mechanism that removes adducts induced by the highly carcinogenic AFB1 (Wilson et al. 2016; Coskun et al. 2019).

The NER mechanism executes the complete excision of the adduct-base-deoxyribose sugar structure, leaving a gap in the DNA backbone that must be repaired by replacing the base-deoxyribose sugar, followed by ligation. Among the repair mechanisms described in this review, NER is the most complex and extensive type of multi-enzyme catalyzed removal and repair of DNA damage of the nuclear genome that dispenses with bulky DNA adducts, such as those formed from BaP and AFB1, that cause significant distortion of the DNA helical structure (Figure 8(D)). During NER-mediated repair, a section of DNA with as many as 25–30 nucleotides, including the damaged base, is removed, and the gap in the DNA helix is sealed by repair synthesis (Fuss and Cooper 2006; Marteijn et al. 2014).

Adducts that occur within actively transcribed genes are preferentially removed *on the fly* by transcription-coupled repair (TCR), whereas adducts in non-transcribed regions are removed later by global-genomic repair (GGR). TCR and GGR are actually sub-pathways of NER as both utilize nearly all of the same enzymes and supporting factors (Ellenberger et al. 2006; Sharma et al. 2009; Friedberg and Zaher 2021). For individuals with hereditary disorders that cause NER deficiency (Giese et al. 1999) or those affecting other DNA repair mechanisms, it is anticipated that these people, if exposed to certain adduct-forming agents in ST products, might be highly vulnerable to cancer, but at present studies in this area are still lacking. Adducts can also cause mutations in genes encoding repair enzymes, such as MGMT (Rohatgi et al. 2005; Sawhney et al. 2007; Lamb et al. 2014), ALKBH2 (Wang et al. 2022), or BER and NER related pathway enzymes (Cleaver et al. 2009) so they do not operate correctly.

3.5. Alterations in cancer-related genes and carcinogenesis

3.5.1. RAS oncogenes

As outlined above, reactive metabolites of certain TSNAs (NNN, NNK), BaP, and AFB1 are all human carcinogens that can form DNA adducts. If not repaired properly, adducts can

cause oncogenic mutations, leading to cancer formation. The *RAS* gene is a key site of oncogenic mutation; indeed, it is the most common oncogene involved in human cancer. The Ras protein normally acts as a guanosine triphosphatase (GTPase) that hydrolyzes guanosine triphosphate (GTP) to guanosine diphosphate (GDP), however, a few mutations can disrupt its proper functioning. Ras functions as an "on/off switch" for certain signal transduction pathways, where Ras-GTP is "on" and Ras-GDP is "off". In its normal functioning, Ras protein intermittently toggles between "on" and "off" as it moderates normal cell proliferation (O'Bryan 2019). Certain mutations cause permanent Ras-GTP to persist, resulting in phosphorylation events permanently switching "on" several sequential downstream proteins in a signal cascade leading to continual gene expression in the nucleus, uncontrolled cellular proliferation, and cancer development (Milde-Langosch 2005; Gurzov et al. 2008; Szalóki et al. 2015; COSMIC Database 2022). Approximately, 20% of all human tumors result from mutations leading to a permanently activated Ras-GTP. In pancreatic cancer, a very aggressive form of cancer, 90% of the tumors have Ras-GTP permanently activated (Spiegel et al. 2014). Inhibition of Ras activity by various drugs is therefore an important strategy for targeting cancer cell growth (Hussain et al. 2020).

Three human *RAS* genes encode four different Ras protein isoforms, namely K-Ras4A and K-Ras4B (produced via alternative splicing), H-Ras, and N-Ras; these all have a length of 188–189 amino acids and a sequence identity of >85%. They have similar but distinct biological functions and are expressed in all mammalian cell lineages and organs. Ras proteins control numerous signaling pathways that impact cell growth/survival, metabolic regulation, apoptosis, oncogenic transcription, and cell migration/adhesion (Tanaka and Rabbitts 2008; Hobbs et al. 2016). Ras is a key component of the epidermal growth factor receptor (EGFR) and Raf-MEK-ERK signaling pathways (McCubrey et al. 2007). Certain adduct-induced mutations can cause GTP-bound Ras to persist and send a continuous downstream "on" signal (Hussain et al. 2020) that triggers constitutive expression of genes, such as *FOS* and *JUN*, that encode transcription factors involved in cancer proliferation that are overexpressed in malignancies (Milde-Langosch 2005; Gurzov et al. 2008; Szalóki et al. 2015; COSMIC Database 2022).

K-Ras is the predominantly mutated isoform in human cancer (75%), followed by N-Ras (17%) and H-Ras (7%), with 98% of these mutations clustering at just three hotspots: G12, G13, and Q61 (Prior et al. 2020). In oral carcinomas associated with tobacco chewing in India, there was a high prevalence of *HRAS* mutations at codons 12, 13, or 61 (Saranath et al. 1991). *KRAS* mutations are associated with high cancer incidence in the biliary tract, and also adenocarcinomas of the lung, pancreas, and large intestines (Tanaka and Rabbitts 2008). In a recent study of KRAS, 81% of the cancer-associated mutations were at codon 12, including variants G12D, G12V, and G12A; 14% at codon 13 (G13D); and 2% at codon 61 (Q61R) (Prior et al. 2020). In *HRAS*, all three hotspots were mutated with comparable frequency. Codon 61 is the predominantly mutated hotspot in *NRAS*. Q61 mutations of *NRAS* are particularly prevalent in melanoma; indeed, 97% of all *NRAS* mutations are found in melanoma. Interestingly, in some cancers, *KRAS* mutations at codon 13 have a worse medical prognosis and outcome than those with codon 12 mutations (Er et al. 2014). While all above-mentioned *RAS* cancer mutations result in hyperactive Ras protein through direct or indirect inhibition of its intrinsic GTPase activity, there is now increasing evidence that *not all mutants are equal* and that the exact phenotypic expression, and hence, the clinical outcome, depends on the particular mutation, the isoform, and the tissue affected (Hobbs et al. 2016).

3.5.2. Tumor suppressor p53

Mutations in the tumor suppressor gene *TP53*, located on human chromosome 17, have also been observed in ST-related cancers, including a significant proportion of oral cancers and pre-cancerous leukoplakia in people in India who chewed tobacco (Saranath et al. 1991, 1999). The p53 protein is arguably the most important human tumor suppressor and has been aptly described as the "guardian of the genome" that detects and repairs DNA damage, or triggers cell death (apoptosis) when damage is too extensive (Lane 1992). Aberrant p53 proteins are found in almost 50 types of cancer (NCBI 2021). The p53 levels are low in normal cells; however, DNA damage or stress increases p53 levels, followed by activation via multiple phosphorylation and acetylation events (Joerger and Fersht 2016). Once activated, the homotetrameric p53 protein binds to specific DNA target sequences and acts as a pluripotent transcription factor that may directly regulate the expression of several hundred target genes and control a wide range of anti-proliferative and homeostatic cell processes (Fischer 2017; Hafner et al. 2019). The mechanisms directly or indirectly controlled by p53 include cell-cycle arrest, DNA repair, apoptotic cell death, bioenergetic metabolism as well as pro-inflammatory and innate immune responses, causing a multi-faceted tumor suppressive response (Vousden and Prives 2009). It was recently discovered that p53 also plays an important role in proper epithelial migration and tissue repair and could accelerate and improve wound repair; so mutations in the p53 protein may hinder these critical processes (Kozyrska et al. 2022).

In normal cells, p53 operates as a barrier against reprogramming toward induced pluripotent stem-cell status. In cancer cells, disruption of p53 functions by mutation, loss of alleles, or protein degradation favors the acquisition of severe phenotypes characterized by increased genome and epigenome plasticity, invasiveness, and capacity to escape cytotoxic treatments (Levine 2020). About 50% of all human cancers carry a loss-of-function mutation in the *TP53* gene. Unlike *RAS* that has three distinct mutational hotspots confined to three residues (G12, G13, and Q61), the *TP53 cancer mutome*–the entirety of *TP53* mutations found in cancers–includes mutations targeting almost every coding base and splice junction of the *TP53* gene (Leroy et al. 2013; Bouaoun et al. 2016). The majority of cancer-associated p53 mutations are missense-mutations (73%), resulting in more than 2,000 different mutant proteins observed in cancer so far, followed by frameshifts (9%) and nonsense mutations (8%), which

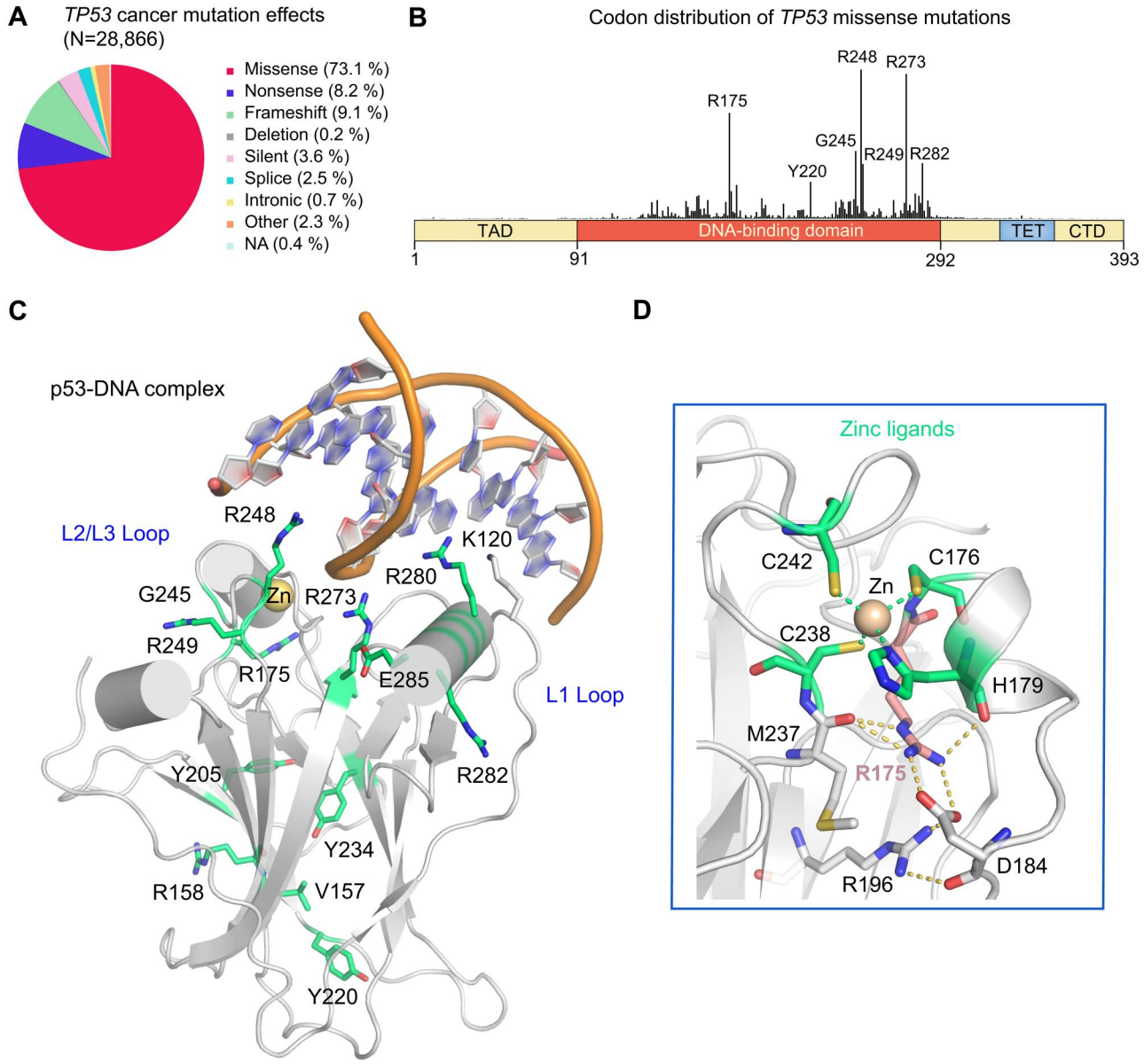


Figure 10. Locations of human p53 cancer mutations. (A) Frequency of different types of somatic p53 cancer mutations in the *TP53* mutation database (release R20, $N = 28{,}286$) of the International Agency for Research on Cancer (Bouaoun et al. 2016). (B) Relative codon distribution of cancer-associated missense mutations in the same database showing that most cancer mutations are located in the DNA-binding domain of the multidomain p53 protein. TAD: transactivation domain; TET: tetramerization domain; CTD: C-terminal regulatory domain. Cancer mutation hotspots are labeled. (C) Structure of the p53 DNA-binding domain in complex with DNA (PDB entry 3KMD, chain B) (Chen et al. 2010). Selected cancer mutation sites discussed in the text are highlighted in green, featuring several essential arginine residues that either form DNA contacts or play a crucial role in stabilizing the p53 structure. (D) Close-up view of the zinc binding site in the DNA-binding domain, highlighting the four zinc-coordinating residues (shown in green) and the structural role of R175 next to the zinc coordination sphere (PDB entry 2XWR, Natan et al. 2011). Figure adapted from reference (Joerger and Fersht 2016).

result in premature stop codons that produce truncated and non-functional proteins (Hainaut and Pfeifer 2016; Donehower et al. 2019) (Figure 10(A)). The p53 cancer mutations have been compiled in several databases, including the UMD TP53 database (Leroy et al. 2013) and the IARC/NCI TP53 database of which the current release R20 from 2019 has been mainly used in this review (Bouaoun et al. 2016; de Andrade et al. 2022). Even though the 393-residue long human p53 protein has several functional domains, including two transactivation subdomains, a proline-rich region, a DNA-binding domain, a tetramerization domain, and a C-terminal regulatory region, most of the cancer mutations cluster in the DNA-binding domain spanning from codon 91 to 292 (Joerger and Fersht 2008, 2016; Tan et al. 2019). Table 1 shows the 30 most frequently occurring somatic *TP53* single nucleotide mutations in cancer and their relative frequency.

When an adduct, derived from an ST product constituent, attaches to a DNA base and is not repaired correctly, it can potentially cause a DNA mutation in which one nucleotide base is permanently substituted for another. A DNA change (e.g. G-to-A) can lead to a permanent amino acid substitution, such as an arginine-to-histidine change in R175H, that can potentially cause the important p53 protein to lose function. Among the top 30 mutations in the *TP53* gene shown in Table 1, DNA base changes occurred mostly at guanine, G-to-A (30%), G-to-T (17%), and G-to-C (3%); but also cytosine,

Table 1. The 30 most frequent human somatic p53 point mutations among all types of cancers in the IARC TP53 mutation database (R20 release).

Rank	Amino acid mutation	DNA base change	Cancer cases	% cancer cases	Mutant class	Protein transactivation class
1	R175H	G → A	1216	4.96	Structural, zinc binding	Non-functional
2	R248Q	G → A	946	3.86	DNA contact	Non-functional
3	R273H	G → A	858	3.50	DNA contact	Non-functional
4	R248W	C → T	765	3.12	DNA contact	Non-functional
5	R273C	C → T	707	2.89	DNA contact	Non-functional
6	R282W	C → T	606	2.47	Structural	Non-functional
7	G245S	G → A	456	1.86	Structural	Non-functional
8	R249S	G → T	440	1.80	Structural	Non-functional
9	Y220C	A → G	402	1.64	Structural	Non-functional
10	R213Stop	C → T	329	1.34	Truncation	NA*
11	R196Stop	C → T	251	1.02	Truncation	NA*
12	V157F	G → T	213	0.87	Structural	Non-functional
13	M237I	G → T	197	0.80	Structural, zinc binding	Non-functional
14	E285K	G → A	186	0.76	Structural	Non-functional
15	H179R	A → G	174	0.71	Structural, zinc binding	Non-functional
16	Y163C	A → G	166	0.68	Structural	Non-functional
17	C176F	A → G	165	0.67	Structural, zinc binding	Partially functional
18	R306Stop	A → G	164	0.67	Truncation after DBD	NA*
19	G245D	G → A	162	0.66	Structural	Non-functional
20	R273L	G → T	155	0.63	DNA contact	Non-functional
21	Y234C	A → G	147	0.60	Structural	Non-functional
22	H179Y	C → T	134	0.55	Structural, zinc binding	Partially functional
23	R248L	G → T	127	0.52	DNA contact	Non-functional
24	Y205C	A → G	122	0.50	Structural	Non-functional
25	S241F	C → T	119	0.49	Structural	Non-functional
26	R158H	G → A	114	0.47	Structural	Non-functional
27	V272M	G → A	114	0.47	Structural	Non-functional
28	Q192Stop	C → T	112	0.46	Truncation	NA*
29	W146Stop	G → A	111	0.45	Truncation	NA*
30	R280T	G → C	108	0.44	DNA contact	Non-functional

DBD: DNA binding domain.
The 2019 release R20 of the TP53 mutation database ($N = 24{,}494$) from the International Agency for Research on Cancer was used; now transferred to the US National Cancer Institute (Bouaoun et al. 2016; de Andrade et al. 2022). Table was produced by Andreas Joerger (co-author on this paper).
*Transactivation potential of nonsense mutants not systematically studied. Predicted to be deleterious.

C-to-T (27%); and adenine, A-to-G (23%). Presence of p53 mutations in pre-malignant oral lesions is low in non-ST users, whereas numerous p53 mutations are found in ST users (Lazarus et al. 1995).

Among amino acids, arginine has unique chemical characteristics, such that the substitution with any other amino acid is one of the main, but not the sole, disruptors of proper functioning of the p53 protein (Borders et al. 1994; Joerger and Fersht 2016). The positively charged guanidinium side chain ($H_2N{=}C(-NH_2)-NH-CH_2-CH_2-CH_2-$) of arginine is slightly longer than the side chain of lysine ($H_3N-CH_2-CH_2-CH_2-CH_2-$). Arginine, which has three nitrogens on its side chain, has the largest capability for hydrogen-bonding and is the most hydrophilic among the 20 amino acids found in proteins. Indeed, arginine plays an indispensable function in stabilizing macromolecular structures by establishing appropriate hydrogen bonds, but also cation–π interactions and salt bridges (Chandana and Venkatesh 2016). Besides its critical interactions within the p53 protein structure, arginine's hydrophilic side chain extends into its aqueous surroundings and interacts with neighboring proteins and negatively charged biomolecules, especially the DNA helix. Substitution of certain arginines with any other amino acid disrupts critical interactions with DNA (Borders et al. 1994; Chandana and Venkatesh 2016; Joerger and Fersht 2016).

Mechanistically, p53 cancer mutations can be divided into two classes: DNA-contact mutants and conformationally unstable structural mutants (Bullock et al. 2000; Joerger and Fersht 2007). Among the top 30 somatic point mutations (Table 1), all of the DNA-contact mutations, seven in all, affect three arginines (R248, R273, and R280) that are crucial for high-affinity, sequence-specific binding of p53 to DNA (Kitayner et al. 2010; Joerger and Fersht 2016). The side chain of R248 on the L3 loop docks into the minor-groove region of target DNAs. R273 interacts with the phosphate backbone of DNA, whereas R280 forms specific hydrogen bonds with a guanine base in the major-groove region of p53 response elements (Figure 10(C)). The main cancer-associated variants at these codons are R248Q, R248W, R273H, and R273C. K120, a lysine residue on loop 1, is involved in binding of proapoptotic target genes, modulated by acetylation of its side chain, but is, surprisingly, a mutational coldspot (Vainer et al. 2016). Structural mutations, such as V157F, R158H, Y220C, R249S, R282W, or E285K (Table 1), are spread across the DNA-binding domain (Figure 10(C)) and induce structural perturbations that reduce the thermodynamic stability of the p53 protein (Joerger et al. 2006; Calhoun and Daggett 2011).

Among the top 30 mutations, structural mutations occur at four tyrosines (Y163C, Y205C, Y220C, and Y234C) that result in non-functional p53 proteins (Table 1). The DNA-binding domain of human p53 has evolved to have a relatively low intrinsic thermal stability (Zhang et al. 2022) and is therefore particularly vulnerable to inactivation by destabilizing mutations that further lower the stability of this protein (Bullock et al. 2000). Even at normal human body temperature, conformationally unstable p53 cancer mutants rapidly unfold and irreversibly aggregate (Friedler et al. 2003; Butler

and Loh 2006; Wang and Fersht 2012; de Oliveira et al. 2020; Billant et al. 2021). Notably, the destabilizing, cavity-creating Y220C mutation, caused by an A-to-G transversion (Bauer et al. 2019, 2020), is the most frequent p53 cancer mutation found in head and neck squamous cell carcinoma, and it is particularly prevalent in oropharyngeal tumors associated with excessive use of alcohol or tobacco (van Kempen et al. 2015). Many p53 cancer mutants have been associated with a gain of novel oncogenic functions, resulting in increased invasion, proliferation, and chemoresistance (Stiewe and Haran 2018; Bargonetti and Prives 2019; Barta et al. 2020; Stein et al. 2020).

A single zinc atom is naturally present in the p53 protein structure, and a particular subgroup of destabilizing structural p53 mutations involve impaired zinc binding (Loh 2010; Blanden et al. 2020). Zinc binding is essential for the stability of the DNA-binding domain and the structural integrity of the L2/L3 loop region of the DNA-binding surface (Figure 10(D)). Mutations impairing zinc binding can directly affect one of the four zinc-interacting residues (C176, C238, C242, and H179) or R175, a neighboring residue. Indeed, R175H is the single most frequent p53 cancer mutation (Table 1) and perturbs zinc binding because of steric clashes between the bulky histidine side chain that is introduced by the mutation and the zinc coordination sphere (Joerger and Fersht 2007).

Some mutations are specific to certain exposures and types of cancer. The structural mutation R249S is one of the most frequent p53 cancer mutations (Table 1) and is highly associated with exposure to AFB1, a crop contaminant common in Southeast Asia and sub-Saharan Africa (Gouas et al. 2009). The guanidinium group of R249 is involved in a hydrogen-bond network that stabilizes the L3 loop, which is key for positioning the neighboring DNA-contact residue R248 for binding to p53 target DNA. Moreover, G-to-T transversions are caused by PAHs from tobacco smoke. PAHs, including BaP, are present in certain ST products made with fire-cured tobacco but also in tobacco smoke (Hainaut and Pfeifer 2001; Pfeifer et al. 2002). BaP can be converted to a reactive metabolite that reacts to form mutational hotspots at several codons, including V157, R158, G245, R248, and R273, in the p53 protein sequence (Barta et al. 2020).

When analyzing cancer mutations at the two hotspot sites R248 and R273 in the latest release of the IARC TP53 database (R20), we found that G-to-T transversions (R248L and R273L) are the most frequent variants in lung cancer. In most other cancers, the G-to-A (R248Q and R273H) and C-to-T variants (R248W and R273C) are the predominant mutant variants at these codons, accounting for four of the five most frequent p53 cancer mutations (Table 1). Common mutational patterns in the DNA-binding domain of p53 in oral cancers of chewing tobacco users include: K132M, R175H, Y205C, M237I, R248Q, R249K, and R273C as compared to V157F, R158L, G245C, R248L, R249M, and R273L in lung cancers associated with tobacco smoke that contains PAHs (Figure A4). As already discussed above, the hotspot mutation R249S is commonly found in liver cancers related to aflatoxin exposure, whereas two uncommon mutations (P152L and V203A) and a hotspot mutation (R248Q) were found in oral cancers related to areca nut exposure (Figure A4).

3.5.3. Genome-wide mutational signatures

With the advent of genome-wide sequencing, analysis of mutational signatures initially identified in *TP53* and *KRAS* genes have been expanded to single nucleotide variations (SNVs) occurring across entire tumor genomes. Currently, the COSMIC mutation database (COSMIC Database 2022) has identified 89 distinct mutational signatures grouped into three main classes: single DNA break signatures (SBSs), 60 signatures; double DNA break signatures, 11 signatures; small insertions and deletions, 18 signatures (Alexandrov et al. 2013). Of these signatures, roughly 20 are associated with defined carcinogens, 25 others with defective DNA processing/repair mechanisms. The etiologies of other signatures are still unknown. Several recent studies have conducted exome-wide sequencing on oral cancers from tobacco chewers (Su et al. 2017; Koo and Ploenzke 2021; Patel et al. 2021; COSMIC Database 2022). A search of the COSMIC database (COSMIC Database 2022) identifies several common signatures, including SBS1, G:C to A:T transitions at CpG sites, and SBS4, G:C to T:A transversions (also called the "tobacco-smoking" signature), which is similar to that seen with exposure to BaP (Barta et al. 2020). SBS29, associated with tobacco chewing, was identified in 39 of 60 oral squamous cell cancers (COSMIC Database 2022). Although the nature of the agents responsible for this signature remains a matter of speculation, the SBS29 mutational profile is consistent with alkyl damage from TSNA induced predominantly on guanine bases (see Sections 3.2 and 3.3) (COSMIC Database 2022).

3.6. Formation of cancer related to smokeless tobacco use

TSNAs, such as NNN and NNK, are organ-specific procarcinogens. Biomarker observational studies showed that ST users have significantly higher levels of nicotine, NNAL, and cotinine in their plasma, saliva, and urine than smokers (Shaik et al. 2019; Chaffee et al. 2020). (*S*)-NNN has been identified as a strong oral cavity carcinogen in ST products (Balbo et al. 2013). In addition, both NNN and NNK induced tumors of the lung, esophagus, nasal cavity and liver in F344 rats as well as of the lung, trachea and nasal cavity in Syrian golden hamsters (Mohamed Anser and Aswath 2014). In ST users, there is a high prevalence of oral, head and neck cancers (Datta et al. 2014). Systematic review and meta-analyses showed that ST use is strongly associated with oral potentially malignant disorders (OPMDs) in South Asia (Khan et al. 2016; Asthana et al. 2019; Khan et al. 2020). Two WHO regions, SEAR and EMR, have high risk of oral and esophageal cancers (Singh 2014; Acharya et al. 2021; Padhiary et al. 2021) with significant positive association with ST use, while the EUR region has high pancreatic cancer rate due to ST (Burkey et al. 2014; Gupta et al. 2018). Apart from malignancy, ST also has negative health implications for the immunological, reproductive, and cardiovascular systems (Willis et al. 2012). Exposure to diverse ST products results in the depletion of endothelial progenitor cells, which may impair endothelium repair. Of note, suppression of the circulating levels of immune cells upon exposure to ST products may increase the susceptibility to secondary infection (Malovichko et al. 2019).

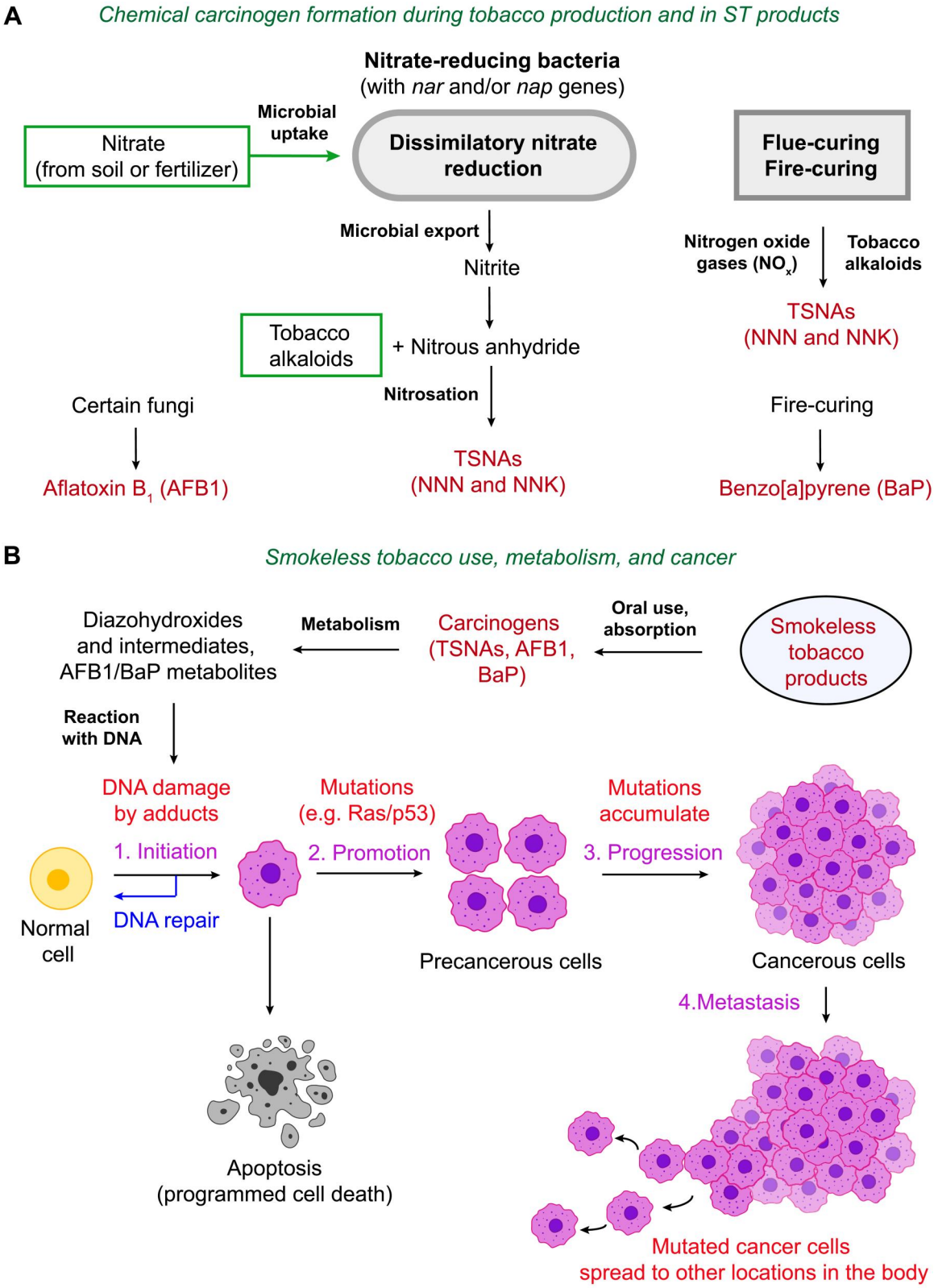


Figure 11. Potential pathways of chemical carcinogenesis related to ST processing and ST product usage. (A) Bacterial generation/export of nitrite followed by abiotic nitrosation. Bacteria expressing dissimilatory nitrate reductases (respiratory or periplasmic) can generate and excrete nitrite that can react with alkaloids to form TSNAs (NNN, NNK). Formation of other potential carcinogens (AFB1, BaP) that can potentially be present in ST products are also shown. Areca nut, another potential carcinogenic ingredient in ST products, is not shown. (B) Absorbed TSNAs can be glucuronidated and excreted (not shown) or converted to diazohydroxides and their intermediates, which form DNA adducts that can lead to deleterious DNA base mutations. Metabolites of other ST-agents can also form adducts leading to carcinogenesis. If adducts cause mutations that remain unrepaired, it can lead to the four stages in chemical carcinogenesis. If DNA damage persists, it can lead to apoptosis where the errant cell dies; or cancer promotion with the formation of pre-cancerous cells. If this process continues further, cancer cells undergo metastasis.

The formation or introduction of carcinogens occurring during ST processing is illustrated in Figure 11(A), whereas the development of a chemically induced carcinoma following ST consumption occurs in a multi-step process shown in Figure 11(B). The first stage of carcinogenesis is initiation when carcinogens in ST modify the molecular structure of DNA by forming an adduct (such as by alkylating agents). Adducts are usually recognized and broken down by body cells (Hecht 2003), but if the adduct escapes detection or repair, then it can lead to mutation of genes causing dysregulation of biochemical signaling pathways associated with cellular proliferation, survival, and differentiation (Li and Hecht 2021). The following step is promotion in which a clonal expansion of the initiated cells occurs due to continuous exposure to a promoter agent, like TSNAs, that speeds up the process of carcinogenesis. Once a tumor has formed, its progression can be further triggered by TSNAs (Becker et al. 2003; Xue et al. 2014). Under these special circumstances, promotion may occur. Promoter compounds can selectively enhance the growth of initiated cells, then cells start to proliferate, forming tumors. This stage is reversible and dependent on the presence of promoter compounds. Next, progression occurs due to repeated exposure of preneoplastic cells to DNA-damaging agents causing activation of pro-oncogenes and inactivation of tumor suppressor genes, thereby causing genomic instability and uncontrolled growth. Further mutations result in higher degrees of independence, invasiveness, and metastasis, conferring permanent genetic growth advantage (Deberardinis and Thompson 2012). Tumor progression is the expression of the malignant phenotype and the tendency for these cells to become more aggressive over time. Lastly, metastasis, a multi-step process that involves the spread of malignant cells from the original site of cancer formation migrating through the bloodstream or lymph system to other parts of the body. Metastasis includes local tumor cell invasion, entry into the vascular system followed by the extravasation of the cancer cells from the circulatory system, and proliferation and colonization in competent organs in the body (Malarkey et al. 2013; Basu 2018).

4. Potential means for minimizing *N*-nitrosamine levels in tobacco production

4.1. Certain bacteria generate and release nitrite that initiates N-nitrosamine formation

As previously established, nitrosamines derived from nicotine and nornicotine (NNK, NNN, and NNAL) (Figure 6) are potent human carcinogens (IARC 2007). As discussed above, previous research has focused on identifying potential problem areas: reactive NO_x gases present during certain types of curing, tobacco species or cultivars with increased alkaloids, leaf nitrate due to absorption of nitrate fertilizers, the presence and activity of certain nitrate-reducing bacteria, and tobacco processing steps (e.g. fermentation and aging) that may increase nitrite accumulation, leading to TSNA formation. Use of ST products results in TSNA that can lead to the formation of reactive metabolites, DNA adducts, DNA base mutations, aberrant oncogenic and tumor suppressing proteins, cancer and, if not successfully treated, ST-related morbidity and/or mortality (Figure 11) (Rutqvist et al. 2011; Fisher et al. 2012; Tobacco Guide 2023).

4.2. Known strategies for decreasing nitrate, certain alkaloids, and microorganisms in tobacco

TSNAs form when tobacco alkaloids react with NO_x gases during some types of curing but also due to the interactions of nitrate, tobacco alkaloids, and certain nitrate-reducing bacteria during processing. Nitrate and tobacco alkaloids, such as nicotine and nornicotine, are generally present at some level in tobacco (Davis and Nielsen 1999) and unlikely to be completely removed. Over the past decades, a number of studies have been published addressing potential TSNA-lowering approaches, including altering processing techniques (Rutqvist et al. 2011; Shi et al. 2013; Gupta et al. 2019), cultivation conditions (Lewis et al. 2012), tobacco lines, and producing areas (Shi et al. 2012) that impact TSNA content. Other strategies have included control of nitrogen fertilization levels (Lewis et al. 2012; Tobacco Guide 2023), implementation of modified curing conditions (Shi et al. 2013), modified fermentation protocols (Fisher et al. 2012), and genetic breeding of tobacco species/varieties (Gavilano et al. 2006; Lewis et al. 2008). Omitting fire-curing prevents TSNA formation but also the accumulation of PAHs, such as BaP, in ST products (Rutqvist et al. 2011; Hearn et al. 2013; Tobacco Guide 2023). Genetic selection of low converter tobacco types, which converts less nicotine to nornicotine, has resulted in decreased NNN levels in tobaccos. Lewis et al. detailed genetic means of targeting TSNA levels by lowering tobacco leaf levels of nitrate or alkaloids, precursors of TSNAs (Lewis et al. 2012). Tobacco plants deficient in nicotine demethylase (NDM) enzyme, which converts nicotine to nornicotine, lack nornicotine (Julio et al. 2008); moreover, when a gene sequence complementary to NDM mRNA is expressed in tobacco plants, it prevents translation of the NDM protein and lowers NNN levels (Lewis et al. 2008).

Deliberate changes in some snus processing and production, especially the elimination of microorganisms via heat treatment, has progressively lowered TSNA levels in these products over recent decades (Wahlberg et al. 1999; Rutqvist et al. 2011; Fisher et al. 2012). When expressed on a wet weight basis, NNN + NNK levels in snus products are generally below 1 μg/g, well below fermented ST products, such as moist snuff and dry snuff with 45–50 μg/g (Richter et al. 2008; Rutqvist et al. 2011; Lawler et al. 2013, 2020) or toombak reaching above 14,000 μg/g (Idris et al. 1991, 1998). Below, we present some existing technologies or approaches, commonly used with food or other orally consumed products, that might aid to minimize the levels of microorganisms or the nitrite by-product.

Several well-established technologies exist in food and pharmaceutical industries that deactivate or eliminate microorganisms by various means, including increased temperature treatment. Pasteurization, commonly used in milk production, is used to process select snus products. For snus, this involves mixing tobacco with water and sodium chloride in enclosed blenders that are injecting hot water and steam to achieve temperatures of 80–100 °C. These temperatures, if maintained for several hours, are sufficient to inactivate microorganisms, but this extended period of heat treatment may not be applicable to all products as it can change the taste or

other characteristics of tobacco and may require modification of processing procedures (Idris et al. 1998; Rutqvist et al. 2011). Of note, some bacteria retain nitrate reducing activity at elevated temperatures (80 – 100 °C) yet that enzymatic activity is lost above 100 °C (Ramírez-Arcos et al. 1998).

Pressurized steam technologies (PSTs) of various types produce high temperatures, pressures, and sometimes flow rates, thus offering a rapid, low-cost, nontoxic, chemical-free microbicidal and sporicidal means of removing or killing microorganisms. PST has proven effectiveness at eliminating microorganisms in food preparation, pharmaceutical, and water treatment facilities. A commercially available pressure washer that can deliver high-pressure hot water and steam can clean and sterilize surfaces, which could include harvested leaves (Figure A5) (CDC 2008; TEMA 2022). These technologies are used to eliminate bacteria on food products, such as herbs and spices (Ventilex 2022). Indeed, a temperature of 132 °C with a pressure of 15 psi can kill bacteria and fungi in 4 min (Alfa 2021). Microwave technology has been successfully used for pasteurizing, sterilizing, and bacterial destruction in production of food, nutraceuticals, pharmaceuticals, and other products. Microwave energy at certain frequencies increases vibrations of water molecules and triggers temperature increases in the aqueous contents of microbial cells, denatures proteins, and other critical biomolecules and ultimately kills microorganisms, including endospore-forming bacilli that can be present in food products (Brinley et al. 2007; Pauly and Paszkiewicz 2011; Chandrasekaran et al. 2013; David et al. 2013). A standard microwave oven can completely inactivate *Geobacillus stearothermophilus* spores (a sterilization indicator) and other undesirable microorganisms in 5 min or less (Rutala and Weber 2019).

Newer high-energy sterilization methods, including electron beam (eBeam) and X-ray technologies, are called "cold pasteurization" because microorganisms are eliminated without increased temperatures that can cause undesirable product changes. The eBeam systems irradiate products with electrons accelerated to 99.9% of the speed of light, whereas X-ray technologies emit high energy X-ray photons (Figure A6). These technologies effectively inactivate bacterial cells, bacterial endospores, fungal mycelium and spores, viruses, and insects in products and are approved by the FDA and USDA for use with orally consumed products. Both eBeam and X-ray energy can pass through packaged products and inactivate bacteria and fungi in the contents by damaging biomolecules in microorganisms that cannot be repaired. At a sufficient irradiation dose, both unpackaged product and finished packages can be sterilized (Miller 2005; Shayanfar and Pillai 2015; Pillai and Shayanfar 2017; Pillai and Pillai 2021).

Because the presence of nitrite is so fundamental to TSNA formation, the addition of nitrite scavenging agents to tobacco can capture nitrite generated and released by bacteria. Nitrite scavenging compounds include vitamin C, caffeic acid, tocopherol, polyphenols, green or Kunlun Tea extracts, and the green tea component epigallocatechin gallate, which are deemed safe for use in food and have also been used in ST products (Choi et al. 1989; Wahlberg et al. 1999; Rundlöf et al. 2000; Rutqvist et al. 2011; Yao et al. 2015) (Table A1). A common chemical feature of nitrite scavenging compounds is the presence of one or more hydroxyl groups that trap nitrite anions (Heijnen et al. 2001). Addition of humectant compounds, such as glycerin and propylene glycol, decreases the water activity of ST products so that microorganisms have less available moisture (Rutqvist et al. 2011). Lastly, refrigeration of products at 4 °C extends product shelf life, slows microbial growth and nitrite-producing activity, slows nitrosation that forms TSNAs, and prevents moisture loss that concentrates TSNA levels in ST products (Djordjevic et al. 1993; Rutqvist et al. 2011).

4.3. *Continued* N-*nitrosamine formation in products after manufacturing*

Processing impacts the constituents of ST products, and factors such as temperature and humidity can impact TSNA levels in the finished ST product. TSNA concentration in tobacco can be increased due to certain tobacco-processing techniques (Chamberlain and Chortyk 1992; Staaf et al. 2005) and storage conditions (Shi et al. 2013). Humidity, temperature, and pH of the stored finished tobacco product also influence TSNAs levels (Stepanov et al. 2015; Wang et al. 2017). Indeed, the storage of ST products for long periods under high humidity and high temperatures increases TSNA levels (Andersen et al. 1991; Hatsukami et al. 2014). As ST products are aged, elevated moisture and insufficient air movement leads to microbial conversion of nitrate to nitrite and increased TSNAs levels (Djordjevic et al. 1993). Storing ST products wet or with high moisture in multi-pack "logs" at temperatures exceeding 37 °C markedly increases TSNA formation (Stepanov et al. 2014). Aging, occurring after ST production, can be slowed down by storing products at cooler temperatures (4 °C) (Djordjevic et al. 1993; Rutqvist et al. 2011).

Although both contain nitrate and tobacco alkaloids, snus and toombak are very different products in terms of their processing and their bacterial content. Toombak is a sun-cured, fermented, and aged product that often contains nitrate, high alkaloid levels due to *N. rustica* content, and individual NNN and NNK concentrations that can exceed 1 mg/g concentrations (Idris et al. 1991, 1998). Idris et al. indicated that TSNA levels in toombak could be decreased by omitting the use of *N. rustica*, and by modifying fermentation and processing of tobacco used to make toombak (Idris et al. 1992, 1998). Others have partially attributed high TSNA levels to fermentation at elevated temperatures, microbial contamination occurring in processing, and prolonged storage (Ahmed and Mahgoob 2007; Ahmed 2013). Recently, several molecular studies of toombak have reported the presence of nitrate-reducing bacteria with respiratory (dissimilatory) nitrate reductases that can contribute to nitrite accumulation in these products (Tyx et al. 2016, 2022; Smyth et al. 2017; Sami et al. 2021).

Lower TSNA levels are found in pasteurized products (e.g. snus) (Lawler et al. 2020) than those found in products processed with fermentation or aging (e.g. zarda, khaini, snuff, toombak, etc.) (Idris et al. 1991; Lawler et al. 2013; Hatsukami et al. 2014). Besides being pasteurized to eliminate microorganisms, snus is not fire-cured, fermented, or aged. One ST company, Swedish Match, recognizing the carcinogenicity of

TSNAs in snus, set into motion remedial actions to eliminate microorganisms and decrease and control the levels of a number of harmful agents (TSNAs, BaP, aflatoxins, etc.) introduced or formed during processing. Starting in the 1980s, this company began implementing new cultivation and processing steps, such as screening soil metals, pasteurizing to eliminate microorganisms, omitting fire-curing, fermentation, and aging steps, and using food-grade ingredients including nitrite-scavenging chemicals (Rutqvist et al. 2011). Swedish-made snus is often produced using air-cured and sun-cured tobacco that is pasteurized prior to further processing. The deliberate use of heat treatment to eliminate microorganisms, omission of fire-curing and fermentative steps (i.e. fermentation, aging), and refrigeration of products after production to slow both microbial growth and reactions all contribute to the consistently low levels of nitrite, various nitrosamines, such as TSNAs and NDMA, mycotoxins (e.g. aflatoxins and ochratoxins), but also VOCs and BaP for some snus products. The type of tobacco used, and the curing method and processing steps utilized are very important factors that determine the levels of carcinogens present in an ST product (Idris et al. 1998; Rutqvist et al. 2011; Hatsukami et al. 2014; Lawler et al. 2020; Swedish Match 2023).

Swedish Match also set maximum permissible levels of toxic metals, nitrite, NNN, NNK, aflatoxins, BaP, and other compounds in their snus products as part of an industry-initiated system, known as the Gothiatek Standard (Swedish Match 2023). These and other harmful compounds are monitored so that their products remain within these pre-defined product limits. Indeed, Swedish Match is the only company with annually published results that achieve the low NNN concentrations of 1 μg per gram of dry weight tobacco or less proposed by the FDA (Federal Register 2017). Recently, Lawler et al. confirmed levels below or slightly above that threshold for NNN in the vast majority of snus products from manufacturers, including Swedish Match (Lawler et al. 2020), whereas moist snuff and dry snuff products from the U.S. (Richter et al. 2008; Lawler et al. 2013) and international products, including khaini and zarda from India and toombak from Sudan (Idris et al. 1991; Stanfill et al. 2011), can far exceed 2 μg/g dry wt. proposed by the FDA (Federal Register 2017).

5. Conclusions

This review illustrates biochemical and chemical events associated with the production of TSNAs during ST processing and mechanisms of carcinogenesis once the compounds enter the body during ST usage. Reactive NO_x gases present during curing can react with alkaloids to form TSNAs in the process of nitrosation. Alternatively, TSNAs can form because of the presence of nitrate and tobacco alkaloids in tobacco leaves and the subsequent enzymatic activity of certain nitrate-reducing bacteria with dissimilatory nitrate reductases, including respiratory nitrate reductases or periplasmic nitrate reductases, that can convert nitrate to nitrite during processing. Nitrite, which is not assimilated in bacterial cells, may be released into tobacco and then react with tobacco alkaloids to form TSNAs via the abiotic process of nitrosation (Spiegelhalder and Fischer 1991; Di Giacomo et al. 2007; Fisher et al. 2012). Indeed, certain nitrate-reducing bacteria, such as *Enteractinococcus*, *Corynebacterium*, *Staphylococcus*, and members of the *Enterobacteriaceae* family, capable of generating and excreting nitrite, have been reported among ST products (Tyx et al. 2016, 2022; Smyth et al. 2017; Rivera et al. 2020; Rivera and Tyx 2021; Sami et al. 2021). Other microorganisms with similar capabilities may be identified as research continues. Whether TSNAs in a given ST product are formed by chemical nitrosation alone, by the action of dissimilatory nitrate reductases or other nitrite-producing enzymes in various microorganisms followed by chemical nitrosation, or a combination, TSNAs accumulate to measurable levels in essentially all ST products that contain processed tobacco (Hatsukami et al. 2014). The main TSNAs formed during the processing of tobacco leaves are NNN and NNK.

Upon ST usage, TSNAs and other carcinogens are absorbed and form carcinogenic metabolites that lead to DNA adducts, which, if unrepaired, can lead to mutations and cancer. It is thought that several thousand adducts are formed in each human cell daily. Fortunately, repair mechanisms exist in the human body that remove most adducts and prevent mutations via single repair enzymes, such as MGMT or ALKBH2, but also via multiple enzyme systems, including NER. Among Ras proteins, mutations are confined to two specific glycines and a glutamine that result in hyperactive Ras proteins that initiate uncontrolled cell growth that can lead to cancer. Also, numerous mutations can inactivate the tumor suppressor p53 (Table 1), a transcription factor that induces the repair of DNA damage, or triggers cell death (apoptosis) when damage is too extensive to repair. It is therefore no surprise that p53 is mutated in about 50% of all human cancer cases. Cancer-associated missense mutations inactivate the p53 protein either by removing crucial, arginine-mediated p53-DNA contacts or by reducing the conformational stability of the p53 DNA-binding domain (so-called structural mutations), causing the protein to rapidly unfold and aggregate in cells (Joerger and Fersht 2016) (Figure 10C). Of note, constituents of ST products or preparations, including TSNAs, BaP, AFB1, or areca nut compounds, each cause mutations at one or more critical arginine residues in the p53 protein found in cancers.

Decreasing exposure to carcinogenic agents due to ST usage and preventing the development of ST-related cancers are important aims. Minimizing the levels of carcinogenic agents in ST products, by altering processing, has been shown in certain Swedish-made snus products for several decades. Snus products, which are pasteurized and omit fire-curing, fermentation, or aging from processing (Idris et al. 1998; Rutqvist et al. 2011), have low documented levels of toxic metals, nitrite, TSNAs, BaP, acetaldehyde, formaldehyde, aflatoxins, and other carcinogens as reported annually (Swedish Match 2023). Indeed, identifying carcinogenic agents or their precursors, implementing processing modifications to decrease those levels, and performing on-going monitoring and adjustments to minimize carcinogen levels has provided a well-tested roadmap leading to ST products with decreased carcinogenic content (Idris et al. 1998; Rutqvist et al. 2011; Gupta et al. 2019; Swedish Match 2023). In particular, the use of pasteurization in early stages of snus production has been a successful approach for eliminating microorganisms such as nitrite-producing

bacteria and mycotoxin-producing fungi, and has resulted in negligible levels of TSNAs and aflatoxins, respectively, in some snus products (Idris et al. 1998; Rutqvist et al. 2011; Lawler et al. 2020; Swedish Match 2023). For ST products not amenable to pasteurization, other proven technologies (e.g. eBeam and X-ray) exist that eliminate microorganisms without increasing the temperature in either of the prepackaged contents or in packaged ST products at the end of production. Moreover, ensuring that toxic and carcinogenic metal or metalloids levels in tobacco are low, omitting fire-curing that contributes to TSNA formation and introduces BaP, other PAHs, and VOCs, and eliminating areca nut as an ST ingredient could remove or decrease the level of other potent carcinogens in ST products. Products processed to eliminate microorganisms, which includes those that are nitrite producing, tend to have very low levels of TSNAs. Other approaches that may have the benefit of decreasing TSNA levels include using tobacco with lower nornicotine content, omitting fermentation and aging of tobacco, refrigerating products, and using nitrite scavenging compounds as additives (Idris et al. 1998; Wahlberg et al. 1999; Rutqvist et al. 2011; Lawler et al. 2020; Swedish Match 2023).

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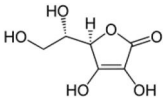
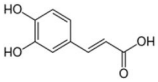
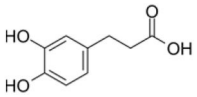
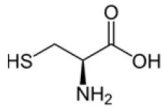
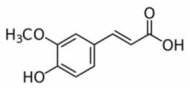
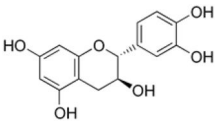
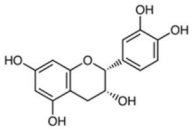
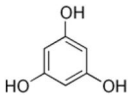
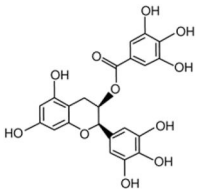
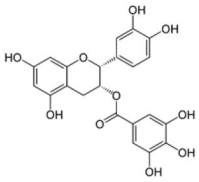
Appendix

Text A1. Regulation of bacterial nitrogen utilization genes

ArnR and GlxR, present in certain *Corynebacterium* species, control the expression of *nar* genes that results in the generation/excretion of nitrite from bacterial cells. The ArnR transcriptional regulator contains two identical monomers with a sensory domain, which can bind or release an FeS cluster, and a DNA-binding domain. Under aerobic conditions, the FeS cluster is bound to the sensory domain, forming FeS-ArnR that remains bound to the promoter region and prevents expression of the *nar* operon (Nishimura et al. 2008, 2011, 2014; Madeira et al. 2019). In the presence of nitrate and anaerobic conditions, some NO is generated endogenously. NO nitrosylates FeS-ArnR, resulting in the loss of NO-FeS from ArnR that is released from the promoter region and permits the expression of the *nar* operon. This regulatory system also includes an activator, GlxR, that binds to the *nar* operator region and promotes *nar* genes expression in response to cyclic AMP (cAMP), due to low O_2 conditions (Nishimura et al. 2008, 2011, 2014). cAMP acts as a secondary messenger of energy status (Nishimura et al. 2008, 2011, 2014). When cAMP binds to GlxR, it associates with the upstream region of the *narKGHJI* operon and triggers the appropriate level of expression in response to nutritional and energy demands (Botsford and Harman 1992; Korner et al. 2003; Kim et al. 2004). Approximately 30% of *Corynebacterium* species, including *C. ammoniagenes*, *C. stationis*, and *C. casei*, contain *narKGHJI*, the genes involved in its regulation (i.e. *arnR*, *glxR*), and *hmp* that converts NO back to NO_3^- (Department of Energy 2022).

Another regulatory system, NreABC, present in some *Staphylococcus* species, controls the expression of *nar* and *nir* genes that allow for the generation/excretion or assimilation of nitrite into biomolecules, respectively. In some *Staphylococcus* species, gene expression of *nirBD* (nitrite reductase), *narGHJI* (nitrate reductase), and *narT* (transporter) are under the control of nitrate regulatory elements (*nreABC*) genes. NreABC consists of a NO_3^--sensing receptor NreA and a two-component O_2-sensing

Table A1. Chemical structures of nitrite scavenging chemicals.

Nitrite scavenging chemicals				
Ascorbic Acid	Caffeic Acid	Dihydrocaffeic Acid	Cysteine	Ferulic Acid
				
Catechin	Epicatechin	Phloroglucinol	Epigallocatechin Gallate (ECGC)[a]	Epicatechin-3-gallate[a]
				

This list of nitrite scavengers shown here was taken from Rundlöf et al. (2000), Choi et al. (1989), and Wang et al. (2017).
[a]Found in green tea extract.

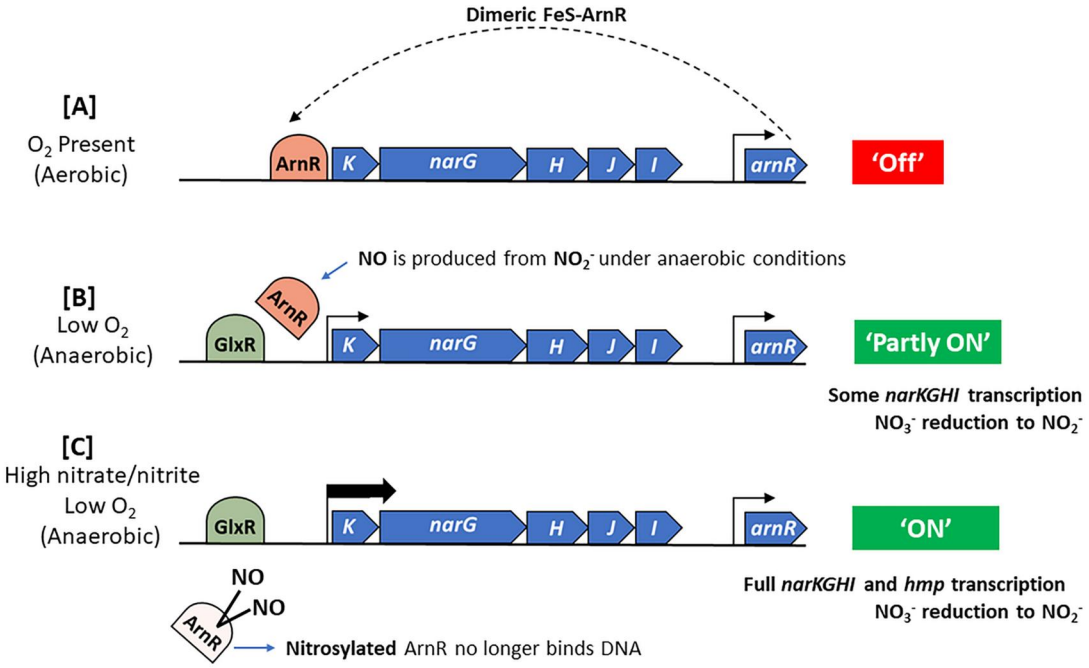


Figure A1. Interaction of ArnR and GlxR transcription regulators with the *narKGHJI* operon in certain *Corynebacterium* species. (A) When O_2 is present, ArnR represses the *narKGHJI* operon (Nishimura et al. 2008, 2014). (B) As O_2 levels decrease, the synergistic behavior of GlxR (an activator) and ArnR (a repressor) cause some nitrate reductase to be synthesized and to convert nitrate to nitrite. When cAMP binds to GlxR, a transcription regulator sensitive to energy status, GlxR binds to the upstream operator and activates the expression of the *narKGHJI* operon, and nitrate respiration produces ATP (Nishimura et al. 2008, 2011, 2014). (C) When O_2 levels are low and nitrate is abundant, some nitric oxide (NO) is generated endogenously from nitrite. Accumulation of NO in the absence of O_2 results in nitrosylation of ArnR, causing a loss in DNA binding and initiation of full activation of *narKGHJI* gene expression (Nishimura et al. 2014). References are listed in the bibliography of the main text.

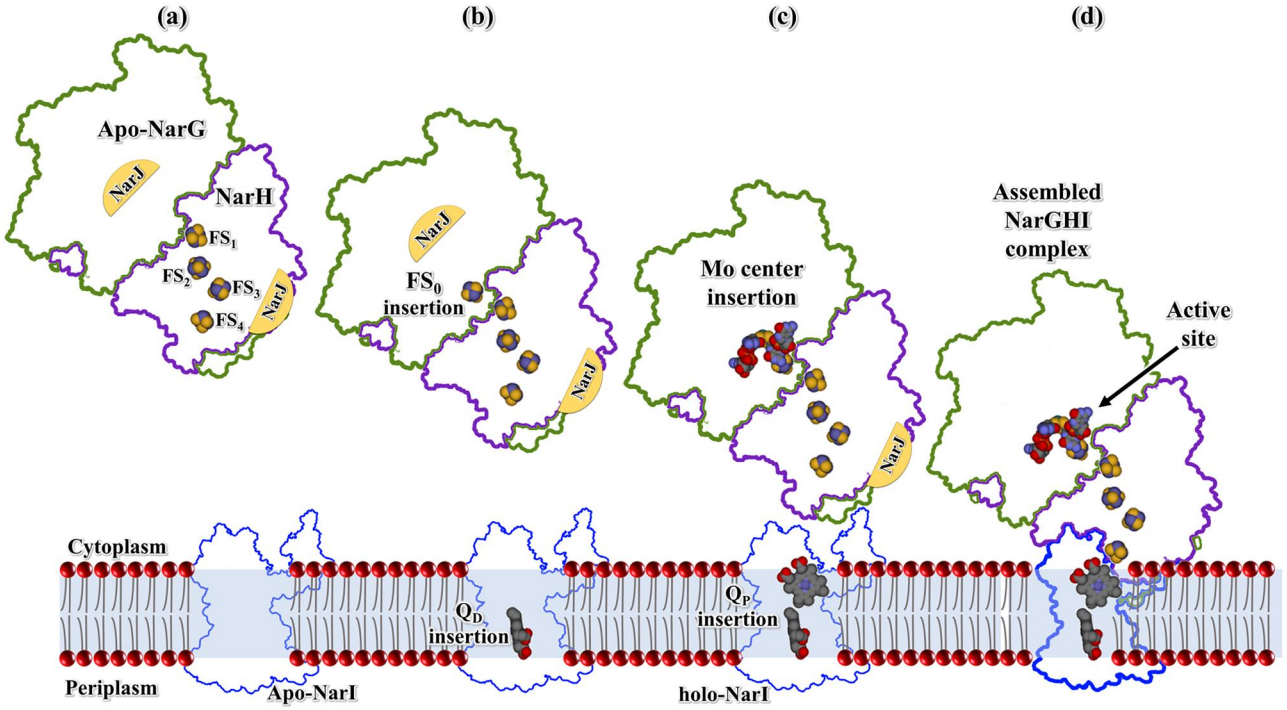


Figure A2. Assembly and maturation of the NarGHI complex is facilitated by the NarJ chaperone. NarG, NarH, and NarI are the α, β, and γ subunits of the respiratory nitrate reductase, respectively; NarJ is not a subunit of the final protein complex but guides the assembly of the NarGHI complex. Prior to the formation of the NarGH complex, the Fe/S center biosynthetic machinery incorporates four Fe/S (FS_1–FS_4) groups into the NarH subunit. Four subsequent steps in NarGHI assembly and maturation are as follows: (a) the NarGH complex is assembled in the cytoplasm with the aid of NarJ, which interacts at several sites with the NarG subunit. (b) The Fe/S (FS_0) group is inserted into the NarG subunit. (c) The molybdenum cofactor (Mo-*bis*PGD) is inserted into NarG, and one NarJ unit is released. (d) The molybdenum and [4Fe–4S] centers are incorporated, then a protein conformational change in NarGH triggers NarJ dissociation, followed by the attachment of NarGH to the NarI subunit so that the entire NarGHI complex is attached to the membrane. NarI maturation is an inner membrane process where the b-type hemes (b_D and b_P) are sequentially incorporated. The structure shown in panel (d) is the fully assembled αβγ monomer.

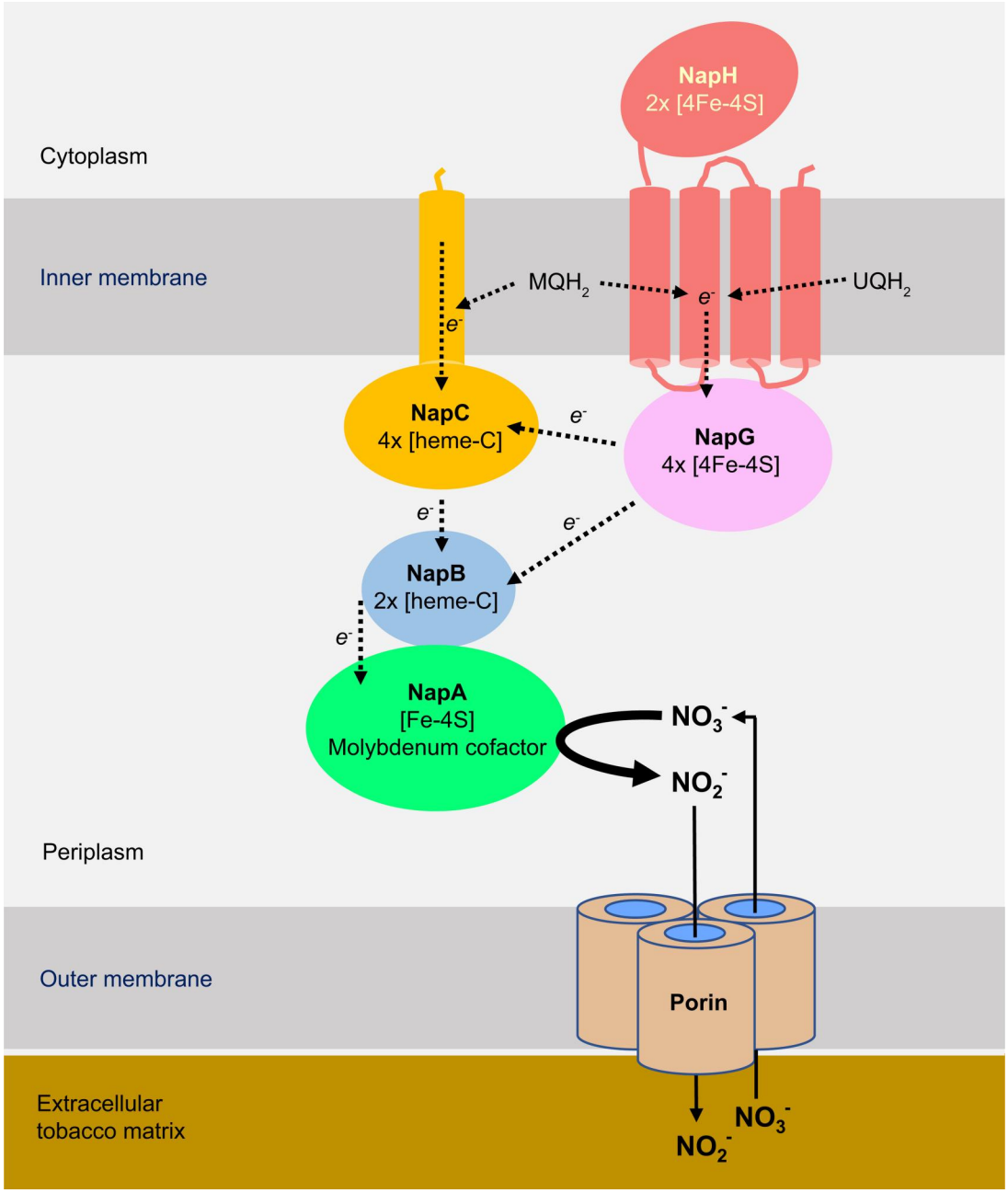


Figure A3. Mechanism of periplasmic nitrate reductase catalyzed nitrite generation in Gram-negative bacteria. The NapA catalytic subunit contains two redox centers (molybdenum cofactor and 4Fe–4S cluster). Various redox-active partner proteins (NapB, NapC, NapG, NapH) localized in the periplasm route electrons to NapA from membrane bound quinone pools (menaquinone, MQH_2; ubiquinone, UQH_2), thus facilitating nitrate reduction to nitrite. Nitrite then passes through outer membrane porins and accumulates in the extracellular tobacco matrix. Nap gene expression and activity are regulated by the presence of oxygen but can function under both aerobic and anaerobic conditions. This suggests that nitrite generation from Nap is functioning during tobacco processing and curing when nitrate is limited.

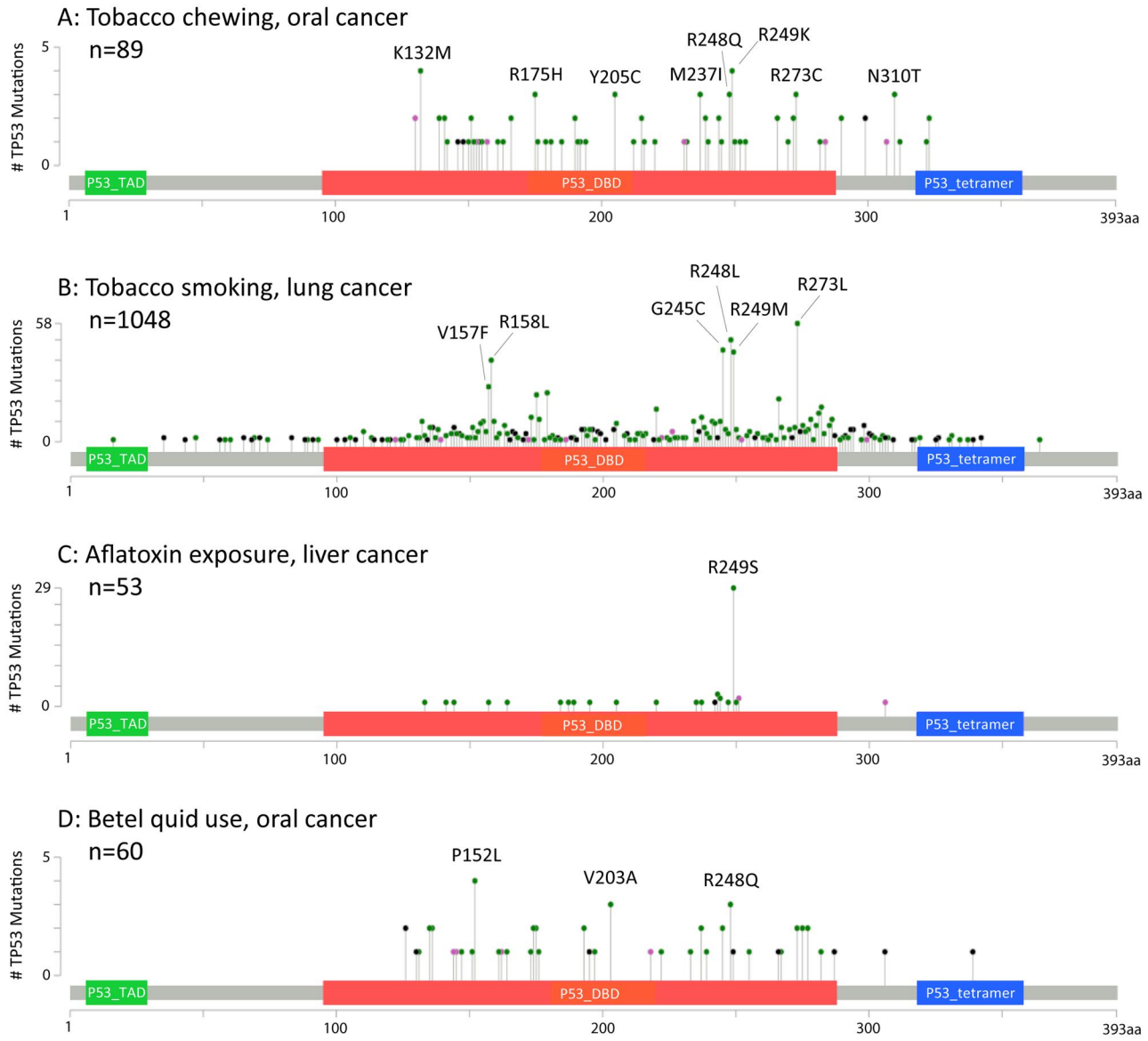


Figure A4. Distribution of tumor *TP53* variants along p53 protein sequence according to exposure risk factors. Data from the R20 version (July 2019) of the IARC TP53 Database were used. Tumors were sorted according to annotations given as the documented exposure in the database. The p53 mutations in: (A) oral tumors with "tobacco chewing" exposure; (B) lung cancers with "tobacco smoking" exposure; (C) liver cancer with "aflatoxin" exposure; (D) oral tumors with "betel quid" exposure are shown. Mutation distributions were visualized using the MutationMapper tool at cBioPortal (https://www.cbioportal.org/mutation_mapper). The most frequent amino-acid substitutions are indicated. Note that exposure annotations in the original IARC dataset and in publications from which this dataset was extracted are incomplete, thus tumors without the exposure annotations used here cannot be ascertained as negative for these exposures.

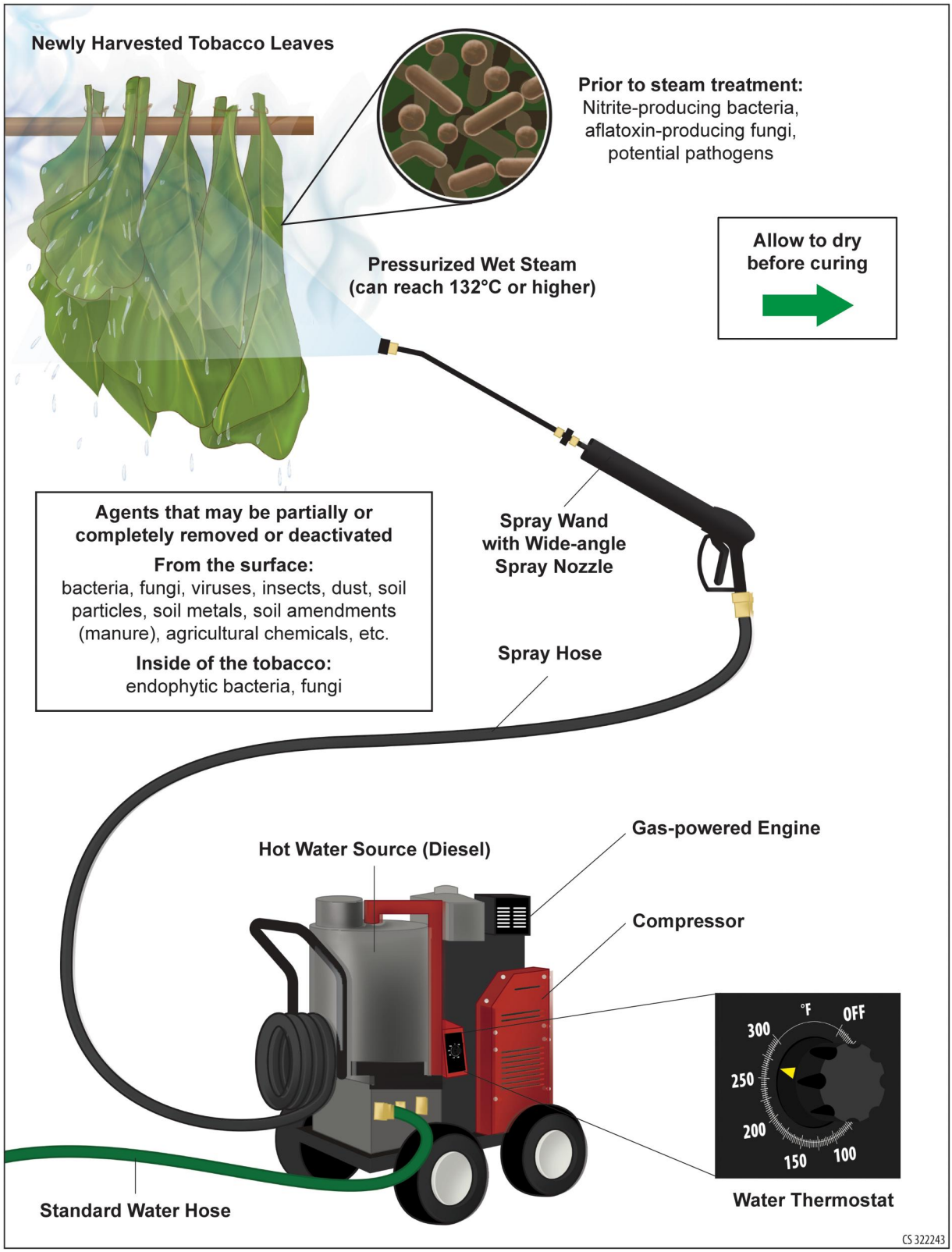


Figure A5. Portable pressurized steam system. This technology uses pressurized hot water to inactivate or remove surface-associated chemicals and microorganisms on newly harvested tobacco. Parameters that may need to be tested: temperature (up to 132 °C), pressure (psi), flow rate (gallons per minutes, GPM), and spray distance. This device uses a standard hose with normal water pressure.

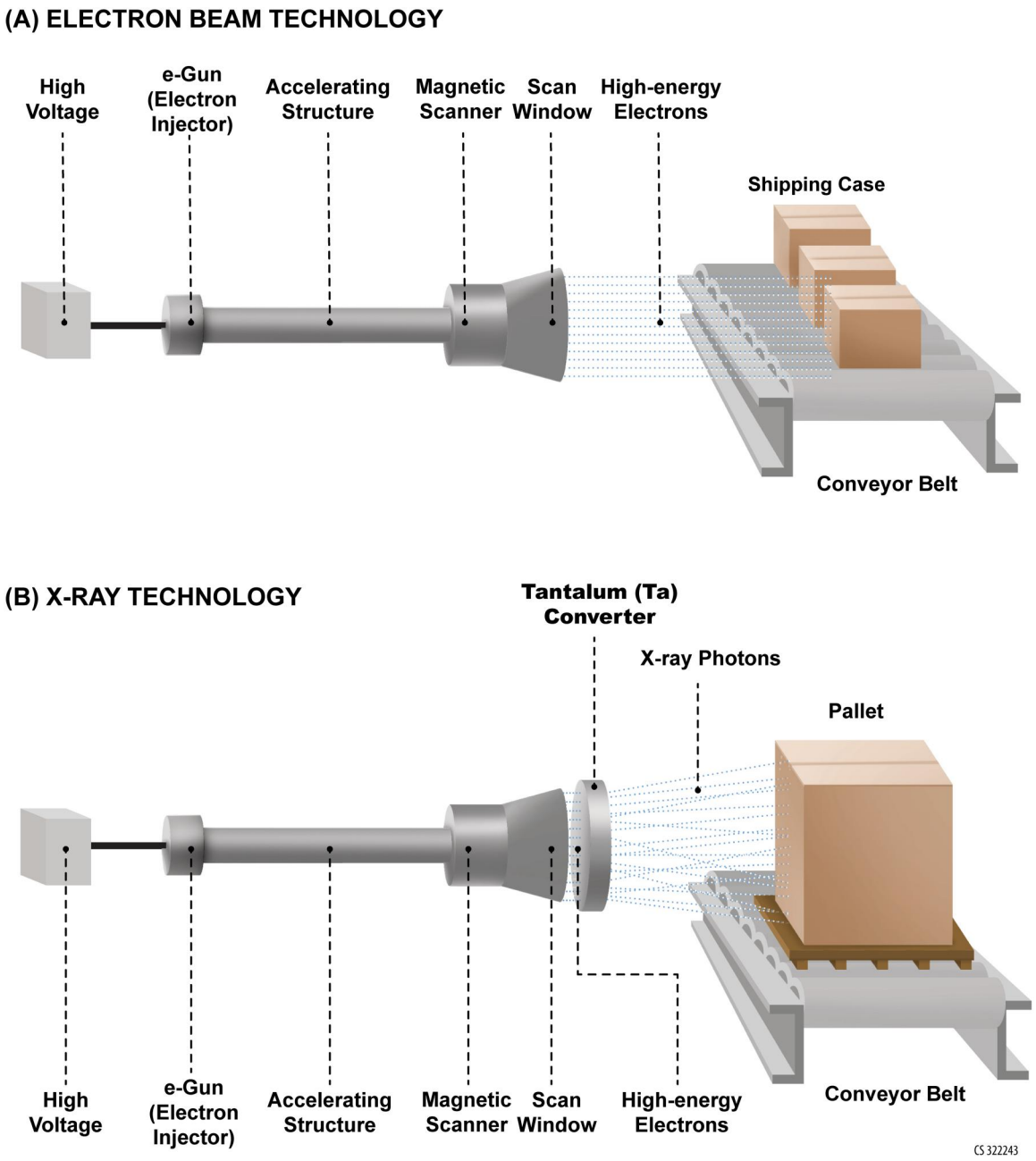


Figure A6. Electron beam (eBeam) and X-ray technologies. These technologies are used to inactivate microorganisms in finished or raw products, such as food, produce, spices, nutraceuticals, pharmaceuticals, and medical devices. Electron beam technology utilizes high-energy electrons, whereas X-ray technology utilizes X-ray photons (that are converted from electrons), which can penetrate entire pallets compared to only shipping cases with eBeam technology. For more information on eBeam see the following references in the bibliography of the main text (Pillai and Shayanfar 2015, 2017; Pillai and Pillai 2021).

system comprised of NreB, a cytoplasmic histidine kinase, and NreC, a response regulator that interacts with DNA promoter regions of several operons (Department of Energy 2022; Fedtke et al. 2002; Kamps et al. 2004). NreB senses anaerobic O_2 levels via a [4Fe–4S] center. NreA and NreB function as an elaborate molecular probe for simultaneous sensing of NO_3^- and O_2 levels, respectively, which then triggers phosphorylation of NreC, leading to appropriate expression of nitrate reductases (NarGHI) or nitrite reductases (NirBD) (Niemann et al. 2014; Nilkens et al. 2014). As research continues, other regulatory systems may be identified in bacteria residing in ST products.