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REVIEW ARTICLE

Tobacco Smoking: Risk to Develop Addiction, Chronic Obstructive Pulmonary Disease, and Lung Cancer

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Abstract: ***Background***: The morbidity and mortality associated with tobacco smoking is well established. Nicotine is the addictive component of tobacco. Nicotine, through the non-neuronal α7nicotinic receptor, induces cell proliferation, neo-angiogenesis, epithelial to mesenchymal transition, and inhibits drug-induced apoptosis.

Objective: To understand the genetic, molecular and cellular biology of addiction, chronic obstructive pulmonary disease and lung cancer.

Methods: The search for papers to be included in the review was performed during the months of July-September 2018 in the following databases: PubMed (http://www.ncbi.nlm.nih.gov), Scopus (http://www.scopus.com), EMBASE (http://www.elsevier.com/online-tools/embase), and ISI Web of Knowledge (http://apps.webofknowledge.com/). The following searching terms: "nicotine", "nicotinic receptor", and "addiction" or "COPD" or "lung cancer" were used.

Patents were retrieved in clinicaltrials.gov (https://clinicaltrials.gov/). All papers written in English were evaluated. The reference list of retrieved articles was also reviewed to identify other eligible studies that were not indexed by the above-mentioned databases.

New experimental data on the ability of nicotine to promote transformation of human bronchial epithelial cells, exposed for one hour to Benzo[a]pyrene-7,8-diol-9-10-epoxide, are reported.

Results: Nicotinic receptors variants and nicotinic receptors upregulation are involved in addiction, chronic obstructive pulmonary disease and/or lung cancer. Nicotine through α7nicotinic receptor upregulation induces complete bronchial epithelial cells transformation.

Conclusion: Genetic studies highlight the involvement of nicotinic receptors variants in addiction, chronic obstructive pulmonary disease and/or lung cancer. A future important step will be to translate these genetic findings to clinical practice. Interventions able to help smoking cessation in nicotine dependence subjects, under patent, are reported.

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1. INTRODUCTION

According to the Global Burden of Disease (GBD) group, tobacco smoking is among the three leading risk factors in terms of attributable Disability-Adjusted Life Year (DALYs), at the global level, that for men is equal to 124.1 million DALYs. Smoking remains among the leading five risk factors for DALYs in 109 countries [1]. The GBD group expects that the burden of tobacco will remain high in future years, in spite of the global decline of tobacco use and second-hand smoke exposure. The burden increase of DALYs is accountable to population growth and ageing, jointly with persistently high smoking habits in several of the most heavily populated countries and in the low social income population [1]. The observation that tobacco habit depends on the environment (i.e. metropolitan or nonmetropolitan areas); level of development, perceived discrimination, gender, economic status, and cultural background emphasizes the requirement of tailored approaches to modify the smoking behavior. The majority of health damages accountable to tobacco smoking are strictly related to tobacco combustion products [2]. Nicotine is the addictive component of tobacco [3-5].

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1.1. Addiction or Substance Use Disorder

The American Society of Addiction Medicine (ASAM) on April 12, 2011, defined addiction as: "*A primary, chronic disease of brain reward, motivation, memory and related circuitry*" [6]. However, "*addiction*" is not considered a specific diagnosis in the DSM-5 - A diagnostic manual used by clinicians that contains descriptions and symptoms of all mental disorders classified by the APA, that recommends the use of the term "*substance use disorder*" (SUD) [7]. The associated symptoms of SUD include impaired control, social impairment, risk use, and pharmacological criteria (i.e., tolerance and withdrawal). Alterations in neurocircuits characterize the complex phenotype of SUD and correspond to three functional domains: 1. binge/intoxication (reward and incentive salience: basal ganglia), 2. withdrawal/negative affect (negative emotional states and stress: extended amygdale and habenula), and 3. preoccupation/anticipation (craving, impulsivity, and executive function: PFC, insula, and allocortex). These three stages nourish into each other making an addiction cycle that becomes more forceful after each cycle, leading to the pathological state of SUD [8].

1.1.1. Nicotine and Nicotinic Receptor

The pyridine alkaloid nicotine is the marking compound of the genus Nicotiana (Family: Solanaceae). Species within the genus Nicotiana contain a high level of nicotine, up to 90-95% of the total alkaloid content. In nature, nicotine is largely used for plant defense; indeed, nicotine poisons AChR resulting toxic to all heterotrophs with neuromuscular junction [9]. Manduca sexta, also known as tobacco hornworm or Carolina sphinx moth, is a nicotine-resistant tobacco-feeding insect. The nicotine-resistance of Manduca sexta reflects the presence of a modified nAChR lacking the amino acid residues required for binding nicotine at the α subunits [10]. It is in homage to Jean Nicot deVillemain, who introduced the queen consort and regent of France, Catherine de Medicis, to tobacco, that the name of the botanical species is Nicotiana and its product nicotine [5]. Native Americans used tobacco essentially during religious ceremonies and for medicinal practices. After its introduction in Europe by the crew of Columbus, the tobacco started to be used as hedonistic purpose until the use of cigarettes exploded during the World War I (1914-18) becoming epidemic [5]. Alton Ochsner, a medical student at the Washington University, after having observed eight cases of lung cancer surgery in six months (in the thirties of the last century lung cancer was considered a rarity), realized that all the patients were heavy smokers who had picked up the smoking habit during World War I [5].

Once in the bloodstream, nicotine rapidly crosses the blood-brain barrier, accumulates in the brain and interacts with nAChR. Nicotine initially acts increasing the white matter integrity then, after chronic use, induces reduction of white matter integrity, probably in connection with activation of nAChR in lightly myelinated tracts [11, 12].

nAChR belong to the cholinergic system (ACh, ChAT, AChE, and mAChR and nAChR). ACh and ACh-synthesizing activity is a very ancient system present in all living organisms from algae and bacteria, including the Archaea [13], to mammals passing through fungi and plants [14]. In mammals, the cholinergic system is expressed in neuronal and non-neuronal cells [14-18]. Eventually, ACh may be considered as a universal mediator involved in the regulation of the organism/cellular homeostasis.

nAChR belong to the cys-loop superfamily of pentameric ligand-gated ion channels [19]. In vertebrates, there are two classes of nAChR, muscle- and neuronal-type, playing crucial roles in neuro-muscular and neuronal transmission, respectively. Mammalian neuronal nAChR consists of eleven subunits ($\alpha2$-$\alpha7, \alpha9, \alpha10, \beta2$-$\beta4$). $\alpha7$ and $\alpha9$ subunits ($\alpha9$ often in combination with $\alpha10$) may form pentameric α-homomeric receptors - i.e. $(\alpha7)_5$, $\alpha2$ and $\alpha6$ require co-expression of at least one β subunit to form functional receptors - i.e. $(\alpha2)_3(\beta2)_2$. The α subunit is the binding-site of ACh, the physiological ligand [19]. In the brain, the two most predominantly expressed receptors are the homomeric $\alpha7$ and the heteromeric $\alpha4\beta2$* nAChR (* denotes the possible presence of other subunits in the nAChR complex, for example, $\alpha5, \alpha6,$ or $\beta3$) (Fig. **1**).

Although the details of the structure and function of the nAChR are beyond the objectives of this review, it is important to remind that homomeric or heteromeric receptors are characterized by important differences in their physiology and pharmacology including sensitivity to nicotine, permeability to Ca^{2+} and propensity to desensitize. In brief, nAChR mediate intercellular communication by converting a chemical signal into a transmembrane ion flux in the postsynaptic cells. At rest, the ion channel is closed, and binding of the agonist (i.e. ACh or nicotine) to the extracellular domain triggers a rapid conformational change resulting in the opening of the transmembrane pore «*gating*» that allows cations to pass inside the cell (Fig. **1**). The cation(s) influx depolarizes the cell membrane and increases neuronal excitability. ACh or exogenous agonists-binding influence the transition rates between three distinct functional states of the receptor: resting, open and desensitized [20]. The transition to non-conducting state is determined either by agonist dissociation, deactivation, or by an agonist-bound conformational change, a non-conducting state, desensitization. Deactivation is the transition from the open state to the resting state associated with dissociation of the agonist. Repeated exposures to nicotine determine an increase in nAChR, upregulation; whereas the mRNA levels of nAChR are unchanged suggesting that upregulation is likely through post-transcriptional mechanisms. Since the nAChR are upregulated, also when the protein synthesis inhibitor cycloheximide is added with nicotine, it is supposed that the existing pool of nAChR subunits can be used for the enhanced stable assembly of the pentamer [20]. Various nAChR subtypes exhibit a diverse range of sensitivities to nicotine and other nicotinic ligand as well as to upregulation. Upregulation seems to be region- and cell-specific. Nicotine binding may activate, desensitize or inactivate nAChR, whereas, chronic nicotine exposure leads to neural adaptations (activation and/or desensitization) that in the case of desensitization can alter neuronal functions interrupting the transmission of ACh [see the special issue "The Nicotinic Acetylcholine Receptor: From Molecular Biology to Cognition" [21].

The neuronal cholinergic system plays a vital role in cognitive functions and the resulting pro-cognitive effects such as improvements in attention, tobacco users [22] have long appreciated working memory and executive processes. Nicotine provokes DA release from neurons of the mesolimbic system and increases the excitatory glutamatergic drive onto DA cell bodies in the VTA [23]. These mesolimbic neurons arise in the

VTA and end in the NAc. Smoking reinforcement may be initially related to the temporarily cognitive improvement, however, after nicotine abstinence, a cognitive disruption happens triggering a "vicious circle" of improvement and disruption that in turn induces dependence [22].

1.1.2. nAChR Subunit Variants

This area of study is nowadays receiving a great deal of research attention after the initial publication of three papers released by Nature [24, 25] and Nature Genetics [26] on 2008 all suggesting a strong association between mutations in the region containing the gene cluster CHRNA5-CHRNA3-CHRNB4. This region is located on the chromosome 15 region q25 (q = long chromosome arm), and encodes the α5, α3 and β4 nAChR subunits, respectively. CHRNA5-CHRNA3-CHRNB4 is involved in nicotine dependence, COPD or lung cancer susceptibility risk (Fig. **2**).

These genetic findings, largely replicated, has been considered as "an exciting convergence of genetic findings, and highlights the potential for research on smoking to inform public health" [27].

A gene cluster is a group of closely related genes that all code for the same function, or variations on the same function. CHRNA5 and CHRNA3 are located in a tail-to-tail configuration on opposite DNA strands. One locus within this cluster, marked by the SNPs rs16969968, has generated particular interest. rs16969968 is a missense variant in CHRNA5 at position 78882925 generating a D398N amino acid change [from aspartic acid (D) to asparagine (N) at codon 398], with the

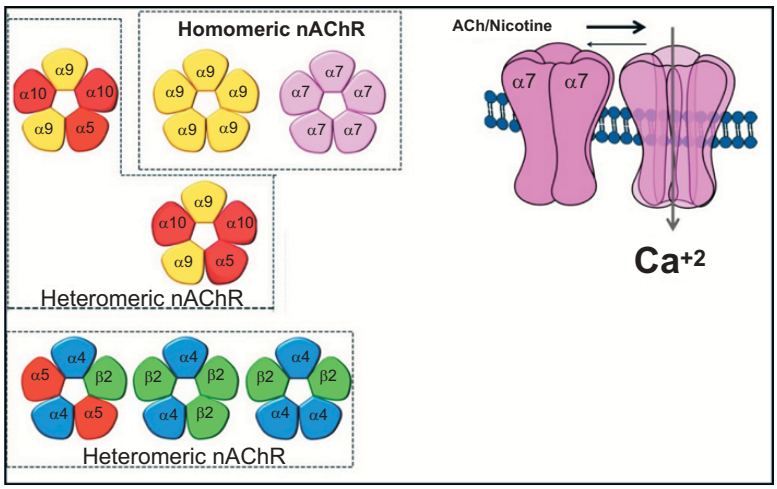

Fig. (1). Homomeric and heteromeric nAChR. In the presence of ligand (ACh or nicotine) the receptor open and Ca^{2+} influx inside a cell. (Images were created using Biomedical-PPT-Toolkit-Suite\06-PPT-Toolkit-Neuroscience [www.motifolio.com].

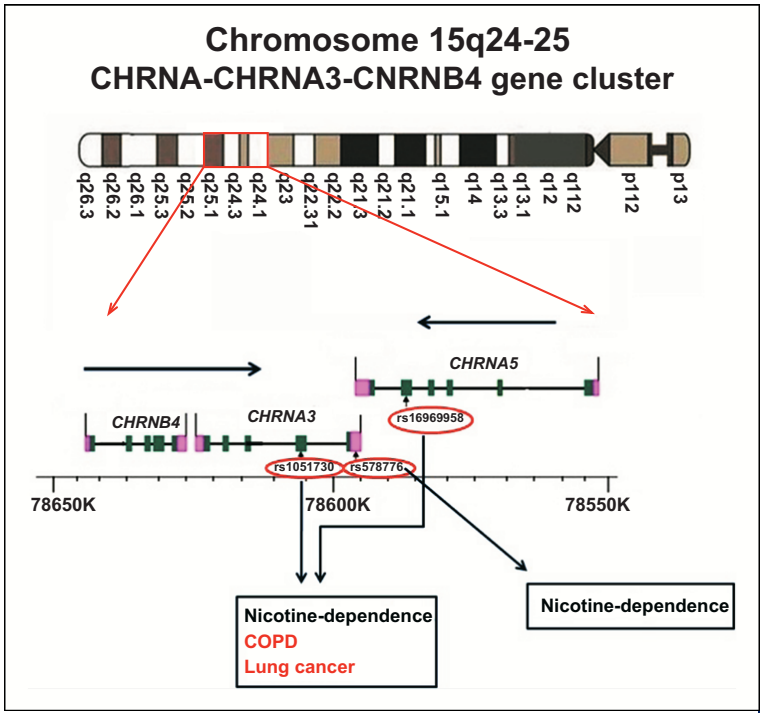

Fig. (2). Schematic picture of the chromosome 15 region q25 containing the gene cluster CHRNA5-CHRNA3-CHRNB4. The horizontal arrows indicate the tail-to-tail configuration on opposite DNA strands of CHRNA5 and CHRNA3 gene. The vertical arrows show the three principal variants involved in nicotine dependence, COPD and lung cancer risk. (Partially Adapted from Wen *et al.* 2016).

minor ''A'' allele being the risk allele and "G" the protective. rs1051730 in CHRNA3 is a coding, synonymous variant. rs1051730 and rs16969968 are in a complete LD (i.e. non-casual alleles association at different loci, not even on the same chromosome); therefore, rs1051730 or rs16969968 may be utilized interchangeably. α5nAChR, as well as β3AChR, are not able to constitute functional receptors, however when incorporated into the pentamer, as accessory subunits, affect the conductance and the desensitization kinetics of the receptor [19]. Accessory subunits play important roles in nAChR function, as they confer unique properties to their parent receptors. Indeed, the resulting α4β2α5 receptors are more permeable to Ca^{2+} than α4β2 receptors and have a higher sensitivity to nicotine [28]. The D398N polymorphism alters the function of α4β2α5 [28]. The presence of aspartic acid (D398) on α4β2α5 causes a diminished agonist-evoked intracellular Ca^{2+} response, reduced Ca^{2+} permeability and enhanced short-term desensitization compared to α4β2α5 possessing asparagine (N398). Recently, it has been reported that addition of either variant of α5 into an α3β4α5 receptor induces likewise effects on receptor pharmacology and function. Nevertheless, the N398 variant induces a reduced response to agonists (ACh or nicotine) under conditions of high external Ca^{2+} and it may lead to distinct downstream cellular signaling. However, the mechanism through which the polymorphism affects receptor function remains unclear [29]. In human D398N allele is associated with heavy smoking [30], early onset of smoking behavior [30], and ''pleasurable buzz'' from tobacco [31]. Moreover, it is a major risk factor for lung cancer or COPD in smokers (see Table **1**); this association is likely to be mediated largely, if not completely, to high exposure to carcinogens contained in tobacco smoke [32]. Human fMRI study have shown a relationship between the presence of D398N risk allele and decreased functional connectivity between the anterior cingulate cortex and the NAc and extended amygdale [33]. These observations sustain, strongly, a primary effect of the gene rather than a secondary effect caused by smoking on connectivity strength [33]. CHRNA5 in exon 5, in which rs16969968 resides, may have different splicing that may decrease the risk of SUD [34, 35]. The shared genetic vulnerability of nicotine and cocaine addictions is particularly interesting as the risk for these two dependencies is conferred by the opposite alleles of rs16969968.

A cluster of nAChR on human chromosome 8p11 (p = short chromosome arm) including CHRNB3-A6 is supposed to be associated with decreased risk for nicotine dependence and increased risk for DSM-5 cocaine use disorder, and there is a nominal association with lung cancer. The α6 subunit expression is detected specifically and almost exclusively in dopaminergic neurons of the *Substantia nigra*, and the VTA. The α6-containing receptors act as enhancers of dopaminergic neurotransmission. Using a transgenic mouse model (gain-of-function α6β2* nAChR -α6L9'S mice) hypersensitive to nicotine and endogenous ACh, it has been shown that increased activity of α6-containing nAChR results in enhanced DA synthesis as well as in increased extracellular DA levels following evoked release [36].

β3, expressed in the human striatum [37], is involved in the conformational changes happening during activation/ desensitization of nAChR and affect both the channel properties and the agonist potency [38]. A large association study examining 348 genes shows that CHRNB3 is one of the most significant gene associated with nicotine dependence [39].

The 8p11 association resembles that of chromosome 15q25; indeed, both regions contain gene clusters that encode for nAChR subtypes, and specific variants in each region are associated with nicotine dependence as well as with lung cancer. Moreover, evidence suggests that nAChR may play a major role in controlling the consumption of addictive drugs other than nicotine, such as cocaine, alcohol, opiates and cannabinoids.

The gene cluster CHRNA5-CHRNA3-CHRNB4A is phylogenetically conserved, and the D398N variant may occur only in humans since this residue is invariant across vertebrate species that all possess an aspartic acid residue (D398) at this location. Although this region is rather homogeneous across three ethnic populations (European, Asian, African-American), the "at risk" allele differs across human populations being predominantly present in populations of European and Middle Eastern ancestry and uncommon or non-existent in populations of African, Asian, or American origin. Otherwise, research implies that any causative variants identified in this gene could be important for almost all smokers, regardless of ancestry [40]. α3α5β2nAChR are expressed at a low level in the basal layer of the pseudostratified normal and stationary bronchial epithelium but at a high level in migrating human bronchial epithelium cells [41]. α3α5β2-nAChR, modulating intracellular Ca^{2+}, contributes to the wound repair of the human bronchial epithelium [41]. *In vitro* experiments using two human lung cancer cell lines that differentiates for CHRNA5 polymorphic status, A549 (rs16969968 GG, D398) and H1299 (rs16969968 AA, N398), show that treatment of either cell lines with nicotine significantly increased invasiveness although, the amplitude of this effect was greater in A549 than in H1299 cells. When CHRNA5 was silenced DNA synthesis was significantly decreased in both cells but was more marked in H1299 (60% decrease) than in A549 (20% decrease). These observations do not explain how polymorphisms of CHRNA5 may affect lung cancer susceptibility [42]. It may be possible that the D398 variant exerts a much potent negative effect on nicotine signaling than N398. Otherwise, polymorphisms in LD with rs16969968 may modulate the expression of CHRNA5 altering the repair of injured human bronchial epithelium. The N398 variant principally happens at low CHRNA5 mRNA expression level. When the D398 occurs at low CHRNA5 mRNA expression level the risk for nicotine dependence and lung cancer is lower than that at high CHRNA5 mRNA expression level [35]. Interestedly, it has been reported that rs1051730 is related to incident COPD, tobacco-related cancers, lung cancer, and smoking quantity and predicts an increased risk of death amongst smokers [43]. rs1051730 is in almost perfect correlation with rs16969968 (CHRNA5) in European populations, thus rs1051730 should be considered as a surrogate marker [44].

At least two different mechanisms of action are associated with the risk of nicotine dependence and lung cancer: (1) a coding variant changes amino acid sequence in CHRNA5 (D398N) and (2) non-coding variants regulating CHRNA5 gene expression.

Recently, it has been reported that CHRNA7 promoter variant rs28531779 (position chr15:g.32322604G > C significantly associated with schizophrenia) is associated with smoking amount [45].

Table **1** summarizes the different CHRNA variants and the correlation with addiction, COPD and lung cancer [40, 46-74].

Table 1. nAChR Genes(CHRNA) Variant and Association with Addiction, COPD and Lung Cancer.

nAChR Subunit Variants	Disease			References
	Addiction	**COPD**	**Lung Cancer**	
CHRNA5-CHRNA3-CHRNB4 gene cluster on chromosome 15q24-25 associated with addiction, COPD and lung cancer				
rs16969968/rs1051780	From GWAS meta-analyses replicated association with cigarettes/day. Receptor modification, sensitization, desensitization. Strong association with tobacco exposure Low effect of peer smoking on nicotine dependence Individuals early-onset smokers with 1 risk allele more likely to be heavy smokers in adulthood Women with the variant AA genotype at significantly increased risk of heavy smoking Increased risk of death amongst smokers	Yes	Yes	[40, 43, 46-55]
rs578775	Women AA decreased risk of heavy smoking			[50]
rs6495309			Yes	[54, 56]
rs578776 **rs1948** **rs684513**	Association with age of first regular tobacco use			[57]
rs1051730, rs8034191			Yes	[58]
rs16969968, rs680244.	The high-risk haplotype increases the risk of cessation failure			[59,60]
rs11634361			Yes	[54]
rs8040868		Yes Protective effect vs severe emphysema		[61 , 62]
rs1051730	Lower likelihood of quitting before hospitalization			[63]
rs2036527, sr5787776, rs11634351,rs11636753, rs1948	Association for traits related to ages at smoking initiation			[64]
CHRNB3-CHRNA6 gene cluster on chromosome 8p11 associated with decreased risk for nicotine dependence and increased risk for DSM-5 cocaine use disorder.Nominal associations with lung cancer				
rs13273442	Nicotine dependence			[65]
rs9298626	Reduced risk for nicotine dependence			[66]
rs6474412	Nicotine dependence		Yes	[24]
rs9298628, rs892413, rs2217732	Association with nicotine dependence in the European American			[67]
rs4950 in the 5' end of CHRNB3	Association with the tobacco adverse and positive subjective factors			[68]
rs10958725, rs10958726, **rs4736835,** **rs6474412, rs4950, rs13280604, rs6474415**	This region is homogeneous across the three ethnic populations			[69]
rs4950	Associated with nicotine dependence			[70]
rs10958726, rs1955186, rs1955185, rs13277254, rs13277524, rs4950	Associated with "dizziness"			[71]
CHRNA2 on chromosome 8p11.21 associated with nicotine-addiction				
rs2472553	Encodes a functional variant in the signal peptide		Yes	[72-74]
rs2292976,rs3735757, rs891398,	Association in the African American sample		Yes	[67]

Briefly, the most important variants are located on:

- CHRNA5-CHRNA3-CHRNB4 gene cluster on chromosome 15q24-25 is associated with addiction, COPD and lung cancer, namely: rs16969968/rs1051780 with addiction, COPD and lung cancer; rs6495309, rs1051730, rs8034191, and rs11634361with lung cancer
- CHRNB3-CHRNA6 gene cluster on chromosome 8p11 associated with decreased risk for nicotine dependence and increased risk for DSM-5 cocaine use disorder. Nominal associations with lung cancer. rs6474412 is associated with lung cancer.
- CHRNA2 on chromosome 8p11.21 associated with nicotine-addiction

1.2. CHRNA7

CHRNA7 (Homo sapiens, also known as NACHRA7) is located on chromosome 15q12.13. CHRNA7 consists of 10 exons, while all other nAChR subunits have six. Exons 5-10 are duplicated [75], and at their upstream, there are three exons partially duplicated with ULK4, a serine/threonine kinase gene mapping at 3p22.1.5, and an additional one of unknown provenience [76]. CHRNA7 is partially duplicated with FAM7A (exons A-E), forming the *chimera* gene CHRFAM7A. CHRFAM7A is present only in humans [77] possibly suggesting an "evolutionary advantage". Simultaneous transcription of CHRNA7 and CHRFAM7A generates α7 and dupα7 proteins, respectively. dupα7 is detected both in neuronal and non-neuronal cells [78-80]. dupα7 may modulate α7-mediated synaptic transmission or cholinergic anti-inflammatory reaction [80]. CHRFAM7A exists in two orientations in respect to CHRNA7 [81]. Expression of CHRFAM7A alone generates protein expression but no functional receptor. Co-expression of CHRFAM7A with CHRNA7 may generate receptors but non-functional (ACh-silent), suggesting that CHRFAM7A is a dominant negative modulator of CHRNA7.

α7nAChR shows peculiar properties different from those of other nAChR.

1) α7 may be considered a primordial type of receptor because, apparently, evolved without additional gene duplications [82]
2) α7 may operate both in ionotropic and metabotropic modes, leading to CICR and G-protein-associated inositol trisphosphate-induced calcium release, respectively. Metabotropic signaling by α7 prolongs the downstream signal of the receptor, most notably Ca^{2+} signaling at synapses [83]
3) α7 shows high permeability to Ca^{2+} [84]
4) α7 activates multiple Ca^{2+} amplification pathways [84]
5) α7 is modulated by the extracellular Ca^{2+}concentrations [84]
6) α7 may bind two-five molecules of agonist and modulates cellular functions *via* phosphorylation and/or via Ca^{2+}-dependent serine/threonine kinases. The occupancy of only one binding site is sufficient for activation. Increasing the number of functional binding sites from one to five does not lead to a concomitant increase in the stability of the open channel [85]
7) α7 is functional without co-assembling with specialized accessory subunits as required by other nAChR subtypes [84]
8) α7 may co-assemble with β2 forming functional α7β2 receptors expressed in human basal forebrain neurons and cerebral cortical neurons [86]
9) Choline is the slightest potent agonist for α7, approximately 10 fold lower than ACh. Nevertheless, choline can produce detectable levels of channel activation at concentrations that are relatively non-desensitizing. α7 current choline-activated may play an important role in Ca^{2+} homeostasis regulation in α7-expressing cells [87]
10) α7 opens rather inefficiently, and, although α7 rapidly desensitizes in the presence of high concentrations of agonist, once desensitized they do not convert to a high-affinity state, as other nAChR [88]
11) The low open probability of α7 can be overcome by positive allosteric modulation and serum factors leading to the generation of excitotoxic currents at physiological temperatures [89].
12) The activity of RIC-3 is critical for the folding, maturation and functional expression of nAChR [90]. α7 needs RIC-3 activity for biogenesis and cell-surface expression. At low levels, RIC-3-depended activity promotes α7 assembly in the ER and surface delivery. At higher levels, RIC-3 suppresses the surface delivery and keeps α7 into the ER [91, 92]
13) α7 require NACHO, a small multi-pass transmembrane protein enriched in neuronal ER, in combination with RIC-3 for proper assembly [93]
14) Since great amounts of α7 remain improperly assembled also in the presence of RIC-3, it has been suggested that additional chaperone such as cholinergic ligands may promote the α7 assembly [94]
15) α7 is palmitoylated with a stoichiometry of approximately one palmitate/subunit during the assembly in the ER [95]
16) α7 regulates NMDAR forming a complex α7nAchR/NMDAR throughout a protein-protein interaction [96, 97]
17) α7 stimulation is needed for NMDA actions [98]
18) α7 promotes the formation of glutamatergic synapses during development [99]
19) The endogenous "prototoxin" LYNX1, belonging to the Ly6 protein family, binds α7 within the extracellular domain, leaving the classical binding site for agonists and competitive antagonists of α7 nAChR unoccupied [100]
20) The prototoxin SLURP-1 is a positive allosteric modulator of α7 [101]

α7nAChR is the major nicotinic subtype highly expressed in the brain (olfactory bulb, cerebral cortex, *hippocampus*, *hypothalamus* and amygdale) as well as in non-

neuronal cells (epithelial, immunological, et cet) [15-18, 102]. Nevertheless, the human α7nAChR 3D structure is still to be elucidated. 3D structure would be of great value in the identification of tobacco constituents with the potential to bind α7nAChR.

1.2.1. CHRNA7 and COPD

COPD is a multisystem disease, with effects beyond the lung that are associated with symptom burden and prognosis [103]. COPD causes chronic airflow limitation, breathlessness, exercise intolerance, cough, difficulty with daily activities, infections and (re)hospitalization [104]. The principal leading cause of COPD is long-term primary or second-hand exposure to cigarette smoke [105]. However, as well as for lung cancer, not all smokers develop COPD, supporting the role of other environmental factors and genetic susceptibility in inducing COPD [106].

The relatively new finding that nAChR are present on non-neuronal cells [107] identified α7nAChR as a crucial player in lung function such as in FEV1 regulation [108, 109]. The role of α7nAChR in airway cells has been discussed largely in a previous review [109].

α7nAChR is a key regulator of the CFTR functional activity in the airway epithelial cells both in the surface epithelium and in submucosal glands. Impairment of airway mucus transport results from dysfunction of CFTR, indeed COPD patients, with a history of chronic smoking, are characterized by an impaired mucus transport. In these patient's chronic exposure to nicotine results in α7nAChR desensitization; consequently, α7nAChR desensitization may contribute to CFTR-related lung diseases in heavy smokers [110]. The lack of functional α7nAChR in the airways leads to squamous metaplasia and loss of ciliary function, alterations observed in patients with COPD [111]. Moreover, α7-nAChR is critical in airway mucous cell metaplasia/hyperplasia and mucus production in response to nicotine [112]. A study, evaluating the effect of a common copy number variation, namely CNV-3956, that duplicates the CHRNA7 gene, performed on 7880 subjects, revealed that ≥ 4-copy of CNV-3956 increased COPD, caused poor lung function, and worsened prognosis [113]. The study estimates that the ≥ 4-copy accounts for 1.56% of COPD heritability representing a possible genetic biomarker [113].

1.2.2. CHRNA7 and Lung Cancer

On 1990, Maneckjee and Minna showed the presence of nAChR on the cell membranes of lung cancer cell lines and found that nicotine partially or totally reversed opioid-induced growth inhibition, establishing that nicotine increased total PKC activity [114]. It is well accepted now that airways epithelial cells express almost every component of the cholinergic system, that α7nAChR controls lung homeostasis and nicotine mediates cell proliferation and tumor progression [17, 115-124].

Among the global actions recommended by the American Association for Cancer Research, there is "*Determine the effects of long-term nicotine exposure on cancer risk, cancer treatment, cancer progression, and survival*" [125]. The average daily intake of nicotine in an inhaler smoker is estimated to be 3.1×10^{-7}M. However, depending on how a cigarette is smoked, it can be as high as 6×10^{-7}M [17, 126]. We report here new unpublished data that chronic administration of nicotine, at 10^{-7}M, fully transforms HBEpC, previously exposed for one hr to 0.1μM BPDE, the major ultimate carcinogen of Benzo[a]pyrene. Table **2** shows that HBEpC, exposed for one h to 0.1μM BPDE and then grown for 16 passages in the presence of nicotine 10^{-7}M, named HBEpC(A), shows the features of transformed cells such as anchorage-independent growth, sustained proliferative signaling, evading growth suppressor, senescence evading, EMT, and evading apoptosis. However, we do not know the ability of these cells to be tumorigenic when transplanted into nude mice, since we decided to not perform experiments in animals for personal ethical reasons.

2. DISCUSSION

Smoking is the second leading risk factor for early death and disability worldwide [127]. Italy has a high SDI for female equal to 17.1 (15.3 to 19.0) and for male equal to 23.2 (21.2 to 25.5) [128]. SDI is a new summary measure of overall development to assess levels and trends in smoking prevalence and attributable burden across the development spectrum.

Tobacco smoking is the main cause of COPD [129]. Others factors such as ambient particulate matter pollution, occupational exposure, and second-hand smoke can cause COPD. It has been speculated that these factors may become a greater cause of COPD in a near future [130]. Of note, COPD in non-smokers may be dissimilar to COPD caused by tobacco smoking in terms of phenotype, comorbidities, and progression [131]. COPD is now one of the most important public health challenge representing the major cause of chronic morbidity and mortality worldwide [127]. It has been reported that in 15 years, COPD will become the leading cause of death [132]. In Italy, chronic lower respiratory diseases (ICD-10 codes: J40-J47) cause 8,324 deaths (5,699 men, 2,625 women) with an ASMR per 100,000 person-years equal to 11.6 for man and 4.8 for a woman (age 30-74 years, period 2012-2014) [133]. Since COPD occurs often in a context of multi-morbidity, COPD remains a growing but neglected global epidemic. Recently, it has been concluded that COPD is under-recognized, under-diagnosed and under-treated resulting in millions of people continuing to suffer from this preventable and treatable condition [134]. The final goal in the treatment of COPD treatment is the prevention of lung function worsening, with consequent symptoms mitigation, including complications treatment. On this contest, pulmonary rehabilitation produces benefits in exercise capacity, symptoms, and health status [135]. Smoking cessation is the most important treatment for smokers with COPD [136].

Globally, lung cancer remains the leading cause of cancer incidence and mortality; among man, it is the first cause in 38 countries and among females in 28. Notably, Denmark, Netherlands, and Hungary are on the top of the list [137]. Lung cancer is now the leading cause of death from cancer in women in the EU-28, thus women die from lung cancer more commonly than from breast cancer in a growing number of countries. In Italy, the estimated number of lung cancer death (hundreds) is 240.3 for man and 104.8 for a woman (for

Table 2. Nicotine Promotes Transformation of HBEpC Previously Exposed for One hr to 0.1μM BPDE.

Cancer Hallmarks	Cells				
	HBEpC	**HBEpC(A)**	**HBEpC(B)**	**HBEpC(C)**	**si-mRNA-a7-HBEpC**
Anchorage-independent growth[a]	No	Yes	No	No	No
Semisolid agar media	(-)	(++)	(-)	(-)	(-)
Spheroids formation	ND	(++)	(-)	(+/-)	(-)
ATP	N.D.	(+++)	(-)	(+)	(-)
Sustaining Proliferative Signaling[b]	No	Yes (++)	No	Yes (+)	No
Doubling time h	36	15	35	28	44
α7-nAChR	(+)	(++)	(+/-)	(+)	(+/-)
Migration and Invasion[c]	N.D.	Yes	N.D.	N.D.	N.D.
Evading growth suppressor[d]	No	Yes	No	Yes	No
p53 and phospho-p53	(-)	(++)	(-)	(+/-)	(-)
pAKT1Ser473/Thr308	(-)	(++)	(-)	(+)	(-)
pMAPKThr202/Tyr204	(-)	(++)	(-)	(+)	(-)
pS6Ser235/236	(-)	(++)	(-)	(+)	(-)
Senescence evading[e]	No	Yes (++)	No	Yes (+)	No
SA-β -Gal activity 16d	(++)	N.D.	(+++)	(+)	(+++)
EMT	No	Yes	No	Yes	No
E-cadherin/ZO-1		(++)		(+)	
Evading apoptosis[f]	No	Yes (++)	No	Yes (+)	No
Cleaved-caspase3	(-)	(-)	(-)	(-)	(-)
Cleaved-PARP	(-)	(-)	(-)	(-)	(-)
BAD	(+++)	(+++)	(+++)	(+++)	(+++)
pBAD	(++)	(-)	(+)	(+)	(+)

Human Bronchial Epithelial Cells "HBEpC", obtained from Cell Applications Inc. (www.cellapplications.com/product no. 502K-05a), were maintained as adherent monolayers in complete Bronchial/Tracheal Epithelial Cell Growth Medium (www.cellapplications.com/product) at 37°C in a 95% air/5% CO_2. Cells were seeded at an initial density of 7.5×10^4 cells/cm^2 and sub-cultured with a 0.25% trypsin–1mM EDTA solution (Sigma-Aldrich, Milan, Italy) when cultures reached 80% confluence. 7.5×10^4 cells/cm^2 semi-confluent HBEpC at 4th passage were treated for 1 h with (a) 0.1 μM BPDE in 0.1% DMSO (BPDE Eagle-Picher Industries,Inc., Chemsyn Science Laboratories, Lenexa, KS] or (b) 0.1% DMSO. Then, cells were washed twice and cultured in the presence or absence of 1.0 x 10^{-7}M Nicotine (Sigma-Aldrich, Milan, Italy). BPDE was handled in accordance with NIH Guidelines for the Use of Chemical Carcinogens.

HBEpC(A): BPDE+continuous treatment with $1x10^{-7}$ M Nicotine for 16 passages

HBEpC(B): BPDE+complete medium

HBEpC(C): continuous treatment with $1x10^{-7}$ M Nicotine for 16 passages

si-mRNA-α7-HBEpC: *a*7silenced cells: BPDE+continuous treatment with $1x10^{-7}$ M Nicotine for 16 passages.

[a]After 16 passages cells were analyzed for their ability to grow in anchorage independent way [cytoselect 96-well transformation assay by Cell Biolabs, Inc] and on Low cell adhesion plates (spheroids formation). ATP formation was calculated by ATP colorimetric assay kit [Bio vision].

[b]Measured with Agilent's Cell proliferation Assay Kit (96 wells)[https://www.agilent.com/cs/library/usermanuals/public/302011-12_Cell_Proliferation_Assay_Kit.pdf].

[c]Measured using CytoSelect 24-well Cell Migration and Invasion assay [fluorometric, Cell Biolabs, Inc]

[d]Measuredusing PathScan cell growth multi-target sandwich ELISA kit [Cell signaling].

[e] Using Cellular Senescence Assay Kit (SA-β-gal Staining) [Cell Biolabs, Inc].

[f]PathSWcan apoptosis using multi-target sandwich ELISA kit [Cell signaling]

α7-nAChR and E-cadherin/ZO-1were evaluated by western blotting.

breast is 125) [138]. The differences in incidence trends, in geographical patterns, in men and in women reflect largely historical, cultural, and regional differences in tobacco smoking [139]. As well as for COPD, 80-85% of lung cancers in Western populations is attributed to smoking, thus the disease might be largely prevented through tobacco control.

Currently, the majority of the general population, at least in the more developed countries, is well conscious of the dramatic increased risk to develop lung cancer caused by tobacco smoking [140]. On the other hand, it is possible to hypothesize that a personal history of smoking (i.e. number of cigarettes per day), and the level of nicotine dependence may influence the perception of this risk among healthy individuals. A recent study shows that people smoking less than 10 cigarettes per day do not see themselves as carrying any risk of lung cancer [141]. Notably, only 38% of patients strongly agree with the statement "*smoking is the cause of most cases of COPD*" [142]. Moreover, a second recent study reports that the 5-year survival probability of a lung cancer patient (at most 15%) is widely overestimated (ex-

ceeding 20%) among smokers and non-smokers [143]. These findings are further supported by recent data showing that people with high nicotine dependence are less likely to quit smoking after lung screening independently of pack-years, in spite of the observation that subjects with high nicotine dependence are more likely to die of lung cancer and all other causes compared with those who are less dependent [144].

Actually, the chromosome 15q25.1 locus is well recognized as a susceptibility region for nicotine addiction, smoking behavior, lung cancer, and, in a lower extent, for COPD (see Table **1** and Fig. **2**). Moreover, it has been reported that epigenetic silencing of nAChR-encoding genes clustered at the 15q25.1 locus may contribute to lung cancer risk [145].

A recent work analyzed a cohort of 1,923 lung cancer cases and 1,977 healthy controls of Italian origin combined with a cohort of 2,995 lung cases and 3,578 controls of European ancestry, to explore the underlying pathways involved in the molecular mechanisms that link variants at the chromosome 15q25.1 locus and lung cancer risk as well increase in lung cancer incidence and development [146]. The findings were replicated with an independent cohort of 18,439 lung cancer cases and 14,026 healthy controls [146]. The findings of the above study suggest that common genetic variations within chromosome 15q25.1 have an effect on lung cancer etiology through the expression/structure and thus the consequent gene functions that encompass the neuroactive ligand-receptor interaction pathway or gated channel activity and connected terms.

It has been shown recently that COPD-risk allele rs12914385: C > T, in CHRNA3, exerts its risk decreasing the DNA methylation level at IREB2 gene, and increasing its expression in COPD patients [147]. This finding supports the hypothesis that the 15q25.1 locus is engaged in the pathogenesis of lung cancer and COPD throughout differential methylation and expression regulation.

All of these results imply the importance to develop new therapeutic drugs able to help smoking cessation according to genetic biomarkers. Table **3** shows new therapeutic approaches now under patent [148-152].

CONCLUSION

The morbidity and mortality associated with smoking is now well established. Smoking cessation is currently the only certain way to reduce the risk of developing COPD or lung cancer. All the findings support the hypothesis that genetic variants are involved in addiction, COPD and lung cancer. On the other hand, nicotine itself induces cell proliferation, neo-angiogenesis, EMS, and inhibits drug-induced apoptosis contributing to lung cancer development and invasion (see Table **2**, Fig. (**3**) and References No: 17, 108-111, 116-124, 153].

Table 3. Interventions Able to Help Smoking Cessation in Nicotine Dependence Subjects, Under Patent.

ClinicalTrials Number / Location	Status	Study Title	Conditions Allocation	Interventions Last Update Posted	References
NCT01780038 University of Nebraska Medical Center Omaha, Nebraska	Completed	Smokers' response to nicotine dependence genotyping	Cigarette smoking nicotine dependence randomized	Behavioral: Participants receive the results of genotyping for rs1051730 No results given February 1, 2018	[148]
NCT00969137 Department of Veterans Affairs West Haven, Connecticut	Completed	Sensitivity to intravenous nicotine: genetic moderators	Nicotine dependence randomized crossover assignment	Drug: Saline Drug: Nicotine April 19, 2017	[149]
NCT01505725 National Institute on Drug Abuse, Biomedical Research Center (BRC) Baltimore, Maryland	Completed	Nicotine reinforcement and smoking-cue reactivity: association with genetic polymorphisms	Nicotine dependence observational	April 5, 2018	[150]
NCT01924468 National Institute on Drug Abuse Baltimore, Maryland	Completed	Brain networks and addiction susceptibility	Nicotine dependence. Impact of rs16969968 on the BOLD fMRI signal and functional connectivity within and between the three networks of interests at rest and during task performance non-randomized intervention crossover assignment	Oral methylphenidate and oral haloperidol September 27, 2018	[151]
NCT01176383 The Integrated Care Partnership The Old Cottage Hospital Epsom, Surrey, United Kingdom	Completed	Impact of a gene test for susceptibility to lung cancer in smokers	Smoking cessation randomized parallel assignment	Respiragene test and risk score May 14, 2014	[152]

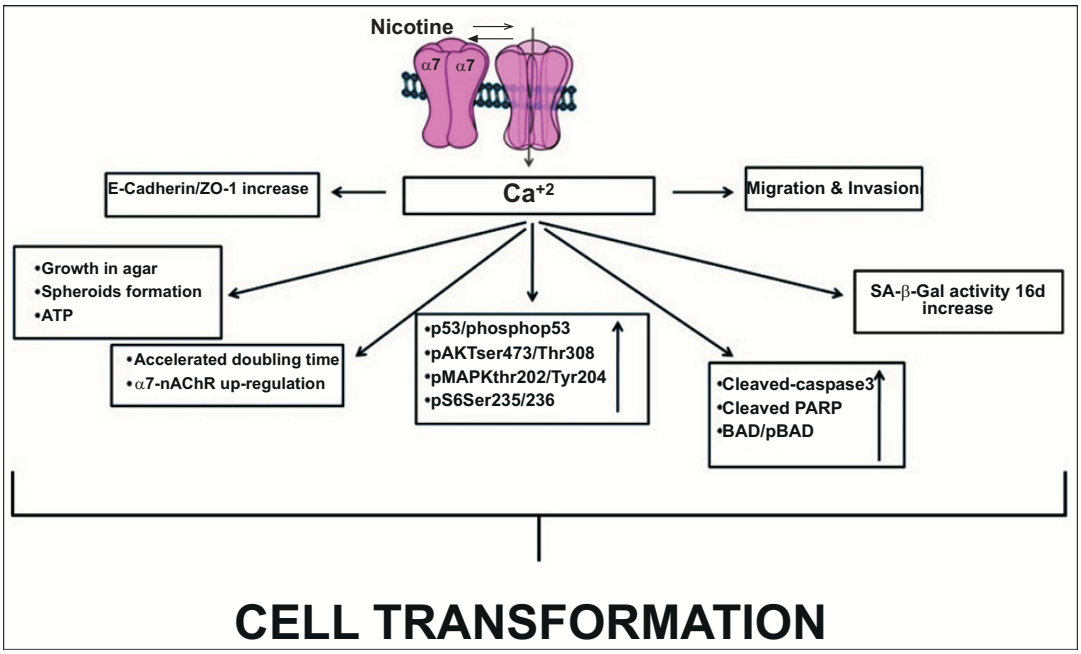

Fig. (3). Cell transformation induced by nicotine via a7nAChR activation.

CURRENT & FUTURE DEVELOPMENTS

Nicotine induces dependence that in turn is responsible for the widespread of tobacco epidemic. Smoking cessation policy and reasonable secondary tobacco as well as environment control approach shall be planned to attempt to reduce the harm from continued exposure. GWAS studies highlight the involvement of nAChR variants in the risk of developing an addiction, COPD and/or lung cancer. A future important step will be to translate these genetic findings to clinical practice with tailored approaches to modify the smoking behavior.

LIST OF ABBREVIATIONS

ACh	=	Acetylcholine
AChR	=	Acetylcholine Receptors
AChE	=	Acetylcholinesterase
ASMR	=	Age-Standardized Mortality Rates
APA	=	American Psychiatric Association
ASAM	=	American Society of Addiction Medicine
N398	=	Asparigine Residue
D398	=	Aspartic Acid Residue
BPDE	=	Benzo[a]Pyrene-7,8-Diol-9-10-Epoxide
CICR	=	Ca^{2+}-Induced Calcium Release
RIC-3	=	Chaperone Resistant to Inhibitors of Acetylcholinesterase
CHRFAM7A	=	Chimeric Gene Formed by CHRNA7+FAM7A
Chat	=	Choline Acetyltransferase
COPD	=	Chronic Obstructive Pulmonary Disease
CNV	=	Copy Number Variation
CFTR	=	Cystic Fibrosis (CF) Transmembrane Conductance Regulator
DSM-5	=	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
DALYs	=	Disability-Adjusted Life-Years
DA	=	Dopamine
ER	=	Endoplasmic Reticulum
EMT	=	Epithelial Mesenchymal Transition
EU-28	=	European Union at 28 Countries
FAM7A	=	Family with Sequence Similarity 7A
FEV1	=	Forced Respiratory Volume in One Second
D398N	=	From Aspartic Acid (D) to Asparigine (N) at Codon 398
fMRI	=	Functional Magnetic Resonance Imaging
GWAS	=	Genome-Wide Association Study
GBD	=	Global Burden of Diseases, Injuries, and Risk Factors Study
HBEpC	=	Human Bronchial Epithelial Cells
ICD10	=	International Classification of Diseases 10^{th} Revision
IREB2	=	Iron Responsive Element Binding Protein 2
LY6	=	Leukocytes Proteins
LD	=	Linkage Disequilibrium
LYNX1	=	Ly6/Neurotoxin 1
mAChR	=	Muscarinic Acetylcholine Receptors
NACHO	=	Nicotinic Acetylcholine Receptor Regulator Chaperone
CHRNA	=	Nicotinic Acetylcholine Receptors Alpha Gene
CHRNB	=	Nicotinic Acetylcholine Receptors Beta Gene
nAChR	=	Nicotinic Acetylcholine Receptors
NMDAR	=	N-Methyl-D-Aspartate (NMDA) Receptor
NAc	=	Nucleus Accumbens

PFC	=	Pre-Frontal Cortex
PKC	=	Protein Kinase C
RIC-3	=	Resistance to Inhibitors of Cholinesterase 3
SLURP-1	=	Secreted Lymphocyte Antigen-6/Urokinase-Type Plasminogen Activator Receptor-Related Peptide-1
SNPs	=	Single Nucleotide Polymorphisms
SDI	=	Socio-Demographic Index
SUD	=	Substance Use Disorder
Dupα7	=	Truncated Subunit Lacking Part of the N-Terminal Extracellular Ligand-Binding Domain of α7 nAChR
ULK4	=	Unc-51 Like Kinase 4
VTA	=	Ventral Tegmental Area

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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