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Review

Cigarette smoke and glutathione: Focus on *in vitro* cell models

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ABSTRACT

Cigarette smoke (CS) is one of the most important preventable risk factors for the development of respiratory diseases, cardiovascular diseases, stroke, and various types of cancer. Due to its high intracellular concentration and central role in maintaining the cellular redox state, glutathione (GSH) is one of the key players in several enzymatic and non-enzymatic reactions necessary for protecting cells against CS-induced oxidative stress. A plethora of *in vitro* cell models have been used over the years to assess the effects of CS on intracellular GSH and its disulphide forms, *i.e.* glutathione disulphide (GSSG) and *S*-glutathionylated proteins. In this review, we described the effects of cell exposure to CS on cellular GSH and formation of its oxidized forms and adducts (GSH-conjugates). We also discussed the limitations and relevance of *in vitro* cell models of exposure to CS and critically assessed the congruence between smokers and *in vitro* cell models. What emerges clearly is that results obtained *in vitro* should be interpreted with extreme caution, bearing in mind the limitations of the specific cell model used. Despite this, *in vitro* cell models remain important tools in the assessment of CS-induced oxidative damage.

1. Introduction

Cigarette smoke (CS) is one of the major lifestyle factors influencing the health of humans. Studies with the largest number of subjects suggest that chronic exposure to CS causes 25% of deaths among humans 35 to 69 years of age (Jha et al., 2013). The premature mortality due to chronic exposure to CS is caused by an increased risk of developing respiratory diseases, cardiovascular diseases, stroke, and cancers of the lung, larynx, mouth, and oesophagus (Colombo et al., 2014). Quitting smoking is the best preventive action and the most effective way to reduce detrimental effects of CS. Unfortunately, despite the well-recognized risks associated with smoking, most smokers are unable or unwilling to quitting smoking (WHO global report on trends in prevalence of tobacco smoking 2015) and the habit remains constantly prevalent worldwide. For this reason, the study of cellular mechanisms of CS-related diseases as well as the tuning of interventional strategies for the reduction of CS-related disease risk are major public health goals.

It is generally accepted that the large number of chemical species contained in CS, such as reactive oxygen species (ROS) (Zhao and Hopke, 2012), saturated aldehydes (*e.g.*, acetaldehyde), and reactive α,β-unsaturated aldehydes (*e.g.*, acrolein, 4-hydroxy-2-nonenal, and crotonaldehyde; Table 1) (Eschner et al., 2011; Schaller et al., 2016; Pauwels et al., 2018; Pennings et al., 2019) induces adverse effects on cells, tissues and organs through oxidative damage to key biological molecules. α,β-unsaturated aldehydes, which have been involved in a number of oxidative stress-induced inflammatory diseases such as vascular diseases (Lee and Park, 2013), can also be generated from oxidatively fragmented oxidized phospholipids as a consequence of cell membrane lipid peroxidation induced by CS-generated ROS (Bochkov et al., 2017). α,β-unsaturated aldehydes form stable covalent adducts with nucleic acids, lipids that contain an amino group, and can modify nucleophilic side chains on amino acid residues, such as the sulfhydryl group of cysteine, the imidazole group of histidine, and the amino

Abbreviations: $(CyS)_2$, cystine; *4-HNE*, 4-hydroxy-2-nonenal; *ABCC*, ATP-binding cassette subfamily C proteins; *ALI*, air-liquid-interface; *CFTR*, cystic fibrosis transmembrane regulator protein; *CS*, cigarette smoke; *CSC*, cigarette smoke condensate; *CSE*, cigarette smoke extract; *Cys*, cysteine; *ELF*, epithelial lining fluid; *GCL*, glutamate cysteine ligase (also called γ-glutamylcysteine synthetase, γ-GCS); *GCLC*, glutamate cysteine ligase, catalytic subunit; *GCLM*, glutamate cysteine ligase, modulatory subunit; *GGT*, γ-glutamyltranspeptidase (also called γ-glutamyl transferase, γGT); *Glu*, glutamic acid/glutamate; *Gly*, glycine; *GPx*, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin (also called thioltransferase); *GS*, glutathione synthetase; *GSH*, glutathione; *GSSG*, glutathione disulphide; *GST*, glutathione-*S*-transferase; *MRP*, multidrug-associated resistance proteins; *NADPH*, nicotinamide adenine dinucleotide phosphate; *PSSG*, *S*-glutathionylated proteins; *ROS*, reactive oxygen species.

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Table 1
Contents of aldehydes in the smoke of some cigarette types, including reference cigarettes used for research.

Cigarette types	Aldehydes	References
2R4F research-reference cigarettes	Acetaldehyde (or ethanal) 622.8 ± 103.3 μg/cig. Acrolein (or 2-propenal) 60.4 ± 6.2 μg/cig. Propionaldehyde (or propanal) 52.6 ± 7.6 μg/cig. Crotonaldehyde (or 2-butenal) 12.5 ± 1.8 μg/cig. Butyraldehyde (or butanal) 11.1 ± 1.6 μg/cig.	Eschner et al., 2011
3R4F research-reference cigarettes	Acetaldehyde (or ethanal) 1555 ± 184 μg/cig. Acrolein (or 2-propenal) 154 ± 20 μg/cig. Propionaldehyde (or propanal) 125 ± 16 μg/cig. Crotonaldehyde (or 2-butenal) 68,8 ± 14.4 μg/cig. Butyraldehyde (or butanal) 88,4 ± 10.7 μg/cig. Formaldehyde (or methanal) 56.5 ± 12.1 μg/cig.	Schaller et al., 2016
Commercial cigarettes Camel Filters	Acetaldehyde (or ethanal) 391 ± 2 μg/cig. Crotonaldehyde (or 2-butenal) 14 ± 4 μg/cig. Formaldehyde (or methanal) 38 ± 33 μg/cig. Propionaldehyde (or propanal) 36 ± 1 μg/cig.	Reilly et al., 2017
Commercial cigarettes L&M	Acetaldehyde (or ethanal) 377 ± 17 μg/cig. Crotonaldehyde (or 2-butenal) 13 ± 1 μg/cig. Formaldehyde (or methanal) 43 ± 24 μg/cig. Propionaldehyde (or propanal) 37 ± 1 μg/cig.	Reilly et al., 2017
Commercial cigarettes Marlboro Red	Acetaldehyde (or ethanal) 407 ± 80 μg/cig. Crotonaldehyde (or 2-butenal) 36 ± 8 μg/cig. Formaldehyde (or methanal) 53 ± 18 μg/cig. Propionaldehyde (or propanal) 36 ± 5 μg/cig.	Reilly et al., 2017
Commercial cigarettes Pall Mall Red	Acetaldehyde (or ethanal) 323 ± 47 μg/cig. Crotonaldehyde (or 2-butenal) 30 ± 1 μg/cig. Formaldehyde (or methanal) 21 ± 4 μg/cig. Propionaldehyde (or propanal) 28 ± 2 μg/cig.	Reilly et al., 2017

group of lysine, resulting in protein carbonylation (Bochkov et al., 2017; Colombo et al., 2010, 2019).

Evidence has shown that chronic exposure to CS can result in systemic oxidants-antioxidants imbalance to the entire organism (Yanbaeva et al., 2007; Shiels et al., 2014), as reflected by the increased levels of several biomarkers of oxidative stress isolated from tissues and biological fluids of smokers and animal models of exposure to CS (Dalle-Donne et al., 2017). The oxidative burden detected in cigarette smokers can occur either *via* direct oxidative damage to biomolecules and/or *via* indirect pathways, such as oxidants-antioxidants imbalance, as reflected by depleted levels of antioxidants, like vitamins A and E (Pasupathi et al., 2009), uric acid (Haj Mouhamed et al., 2011), cysteine (Moriarty et al., 2003), and glutathione (Moriarty et al., 2003; Pasupathi et al., 2009; Pannuru et al., 2011; Fratta Pasini et al., 2012).

Glutathione (γ-glutamyl-L-cysteinylglycine, GSH) is one of the key players in several enzymatic and non-enzymatic reactions necessary to protect cells against CS-induced oxidative stress. GSH has antioxidant properties since the thiol group of its cysteine moiety is a reducing agent and can be reversibly oxidized to its disulphide forms — *i.e.*, glutathione disulphide (GSSG) and *S*-glutathionylated proteins (PSSG) —— although, its role as an antioxidant in cells is not direct but predominantly mediated by enzymatic reactions to which it takes part (Deponte, 2017). Furthermore, the γ-bond between glutamic acid and cysteine confers its insusceptibility to hydrolysis by most of the cellular proteases and peptidases, except γ-glutamyltransferase, localized to the plasma membrane and acting extracellularly, and the enzymes from the ChaC family (Bachhawat and Kaur, 2017) (Fig. 1). Within the majority of human cells GSH is ~1–2 mM, while in hepatocytes, which are the major producer and exporter of plasmatic GSH, its concentration can reach ~10 mM (Giustarini et al., 2017). Despite its exclusive cytoplasmic synthesis, cellular GSH is not only abundant in the cytosol (1–11 mM) but is distributed in intracellular organelles, including the nucleus (3–15 mM) and mitochondria (5–11 mM), where it is the major soluble antioxidant and exists predominantly (95–99%) in the reduced form due to NADPH-dependent GSSG reductase (Valko et al., 2007). Together with other antioxidants (*e.g.*, ascorbic acid, albumin, and α-tocopherol), GSH is also present at high-concentrations in the respiratory tract lining fluid, which covers the epithelium throughout the airways (Halliwell and Gutteridge, 2007).

Many different cellular models have been used in *in vitro* exposure experiments to study the pathological processes involved in CS-related human diseases. Even if translation of the outcomes from the research laboratory to the bedside is often challenging, *in vitro* models are still one of the pillars of contemporary research. The goal of an *in vitro* cell model is to simplify the experiment, limiting experimental variables and to provide new information on molecular and functional differences between cells of smokers and non-smokers. Furthermore, though a full abolition of animal models is highly unlikely in the near future, the use of cell models will gain more and more importance as a result of global diffusion of initiatives like the 3Rs (refining, reducing, and replacing animal models) (Flecknell, 2002).

In this review, we will describe *in vitro* human cell models used to assess cellular responses to CS, focusing our attention mainly on the role of GSH, its disulphide forms (GSSG and PSSG), and GSH-aldehyde adducts (GSH-conjugates). We will also highlight the pros and cons of the use of these model systems, emphasizing limitations that can make it difficult to extrapolate results from *in vitro* studies to the complex system of the human organism.

2. Intracellular biosynthesis of glutathione

GSH is synthesized within the cytosol of all mammalian cells, although the rate of its synthesis, turnover, and intracellular concentrations differ among the various cell types and tissues of the body. Unlike most peptides and proteins, GSH is not synthesized by ribosomes but its synthesis is catalysed, from its constituent amino acids, by two ATP-dependant cytoplasmic enzymes in two sequential reactions. The first reaction, catalysed by glutamate cysteine ligase (GCL) (Fig. 1), leads to the synthesis of γ-glutamyl-cysteine from glutamate and cysteine in the presence of ATP and is the rate-limiting step in the GSH biosynthesis (Lu, 2009). The second reaction is catalysed by glutathione synthetase (GS) (Fig. 1) and involves the ligation of γ-glutamyl-cysteine to glycine in another ATP-dependant reaction to produce γ-glutamylcysteinylglycine (or glutathione). GCL is a heterodimeric holoenzyme complex consisting of a catalytic subunit (GCLC) and a regulatory subunit (GCLM) (Fig. 1). GCLC contributes all the enzymatic activity, contains all the substrate and cofactor binding sites of GCL and is regulated by non-allosteric feedback inhibition exerted by GSH. GCLM modulates GCLC activity and affinity for substrates and inhibitors (Lu, 2009).

Although the structure and activity of GCL have been extensively studied and defined in the 1990s (see Lu, 2009 for a complete overview), more recent studies have expanded the knowledge on the regulation of the expression and activity of the two enzymatic subunits. Increased GCL activity following numerous oxidative cellular insults shows that the induction of the enzyme acts as an adaptive response to oxidative stress (Krejsa et al., 2010). The genes of both GCLC and GCLM are under the transcriptional control of several transcription factors, including the redox-sensitive factor nuclear erythroid-related factor 2 (Nrf2), which induces GS as well (Moinova and Mulcahy, 1999) and whose activity decreases with age (Suh et al., 2004). Some reviews have provided an excellent summary of the various transcriptional inducers of GCL (Maher, 2005) and of the major post-translational regulatory mechanisms, such as phosphorylation, myristoylation, and caspase-mediated cleavage, of GCLC (Franklin, 2009).

When GSH is consumed and feedback inhibition is lost, availability of cysteine as a precursor can become the limiting factor for GSH syn-

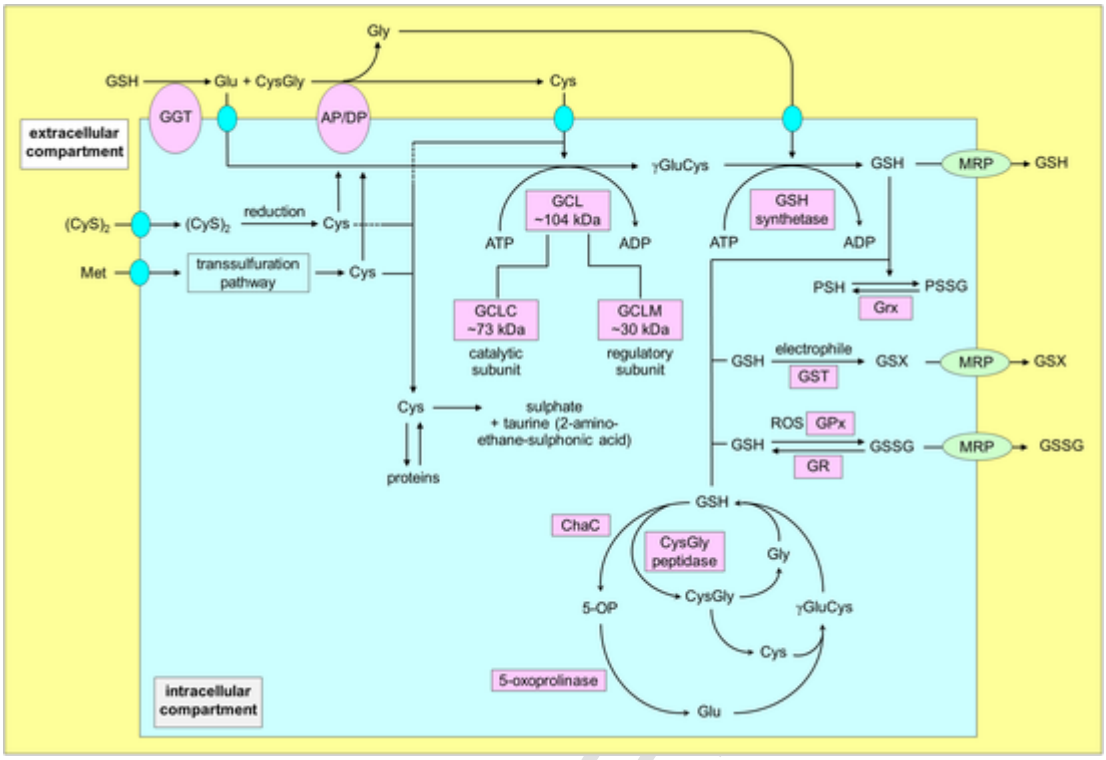

Fig. 1. Metabolism of GSH and its contribution to cellular redox homeostasis. The hydrolysis of extracellular GSH is catalysed by the ectoenzyme GGT, which releases Gly and CysGly. CysGly is broken down by AP or DP to generate Cys and Gly. The three amino acids can then be taken by the cell through amino acid transmembrane transporters. In humans and mouse, two ChaC-like proteins are involved in the intracellular degradation of GSH, namely the γ-glutamylcyclotransferases ChaC1 and ChaC2. ChaC2 is constitutively expressed and active, being so responsible for the continuous slow degradation of GSH (used primarily for Cys cellular supply), whereas ChaC1 is upregulated during development and during stress conditions. *De novo* GSH synthesis within the cell can occur from its three precursor amino acids in two sequential reactions catalysed by two ATP-dependent, cytosolic enzymes. The first enzyme is γ-glutamyl (glutamate)-cysteine ligase (GCL) that combines glutamate and cysteine: it is generally rate limiting. The second enzyme is glutathione synthetase (GS), which adds glycine, yielding GSH. Because glutamate and glycine occur at relatively high intracellular concentrations, cysteine is limiting for GSH biosynthesis.Transsulfuration pathway (also called cystathionine pathway) is particularly active in the hepatocytes, which allows the liver to use Met effectively for GSH synthesis. After its synthesis, some GSH is delivered into specific intracellular compartments, but much of the GSH is retained into the cytoplasm or delivered to extracellular compartment through MRP. Four distinct classes of GSH-dependent enzymes contribute to cellular redox homeostasis: GPx, GR, GST and Grx. GPx catalyses the reduction of a range of organic or lipid hydroperoxides to water or corresponding alcohols, respectively, with GSH as a reducing substrate. In this reaction GSSG is formed. GSSG can then associate with protein sulphydryls to produce PSSG, it can be exported from the cell through MRP, or it can be reduced back to GSH by GR at the expense of NADPH. All this occurs in an attempt to maintain the high intracellular GSH to GSSG ratio. In addition to being a reductant in GPx reaction, GSH can also form conjugates (GSX) with various electrophilic compounds, mainly by the action of GST. Both GSH and GSX can be exported from the cell through specific MRP. *5-OP*, 5-oxoprolina, *GSH* glutathione, *GSSG* glutathione disulphide, *GSX* glutathione conjugates, *PSSG* protein glutathione mixed disulphides (*S*-glutathionylated proteins), *PSH* protein sulphydryls, *GCL* glutamate cysteine ligase, *GGT* γ-glutamyltranspeptidase, *GPx* glutathione peroxidases, *GR* glutathione reductase, *Grx* glutaredoxins, *GS* glutathione synthetase, *GST* glutathione *S*-transferases, *AP/DP* aminopeptidase/dipeptidase, *MRP* multidrug-associated resistance proteins, *ROS* reactive oxygen species.

thesis (Biolo et al., 2007; Lu, 2009). Cysteine (Cys) is derived from the diet, from protein breakdown, and from methionine *via* homocysteine by the transsulphuration pathway that is particularly active in the liver, but is also present in the kidney, in the intestine, and in the pancreas (McPherson and Hardy, 2011). In the liver, ~50% of the Cys in GSH comes from methionine *via* the transsulphuration pathway. Cys differs from other amino acids because its reduced form is predominant inside the cell whereas its disulphide form, cystine ($(CysS)_2$), is predominant outside the cell. Once $(CysS)_2$ enters the cell, it is rapidly reduced to Cys (Lu, 2009). Inside the cell, the majority of Cys is incorporated into GSH, some is incorporated into protein, depending on the need of the cell, and some is degraded into sulphate and taurine (Fig. 1).

3. Compartmentalization, export, and catabolism of glutathione

Although the majority of the GSH is retained in the cytoplasm, after synthesis GSH is also distributed between intracellular organelles such as mitochondria, the nucleus, and the endoplasmic reticulum. The charged nature of the GSH molecule at physiological pH does not allow the passive diffusion across the intracellular membranes. Rather, GSH must be transported actively or in exchange for another anion. Several transporters have been proposed to be involved in the transport of GSH into organelles, among which there are the dicarboxylate carrier and the 2-oxoglutarate carrier on the inner mitochondrial membrane (Lash, 2006), although their involvement has recently been questioned (Booty et al., 2015), the Sec61 protein-conducting channel on the endoplasmic reticulum (Ponsero et al., 2017), or the ryanodine receptor type 1 (RyR1) on the sarcoplasmic reticulum of striated muscle fibres (Bánhegyi et al., 2003). The outer mitochondrial membrane is rich in porins, which form aqueous channels through the lipid bilayer and allow diffusion between the inner mitochondrial membrane and cytosol of molecules smaller than ~5 kDa, including GSH. The mechanism of GSH transport and sequestration into the nucleus is at present unknown, though some evidence suggests the involvement of a mechanism regulated by Bcl-2 proteins (García-Giménez et al., 2013) and the engagement of the nucleoporin ALADIN (Queval and Foyer, 2014). In particular, Bcl-2 proteins harbor a BH-3-domain, which binds GSH. Bcl-2 proteins also play a major role in the maintenance of mitochondrial GSH levels: in fact, Bcl-2 proteins shuttle GSH across the mitochondrial inner membrane (Zimmermann et al., 2007) and Bcl-2 is an interacting partner for the 2-oxoglutarate carrier (Wilkins et al., 2012).

Much of the intracellular GSH is exported from the cell to extracellular fluids, such as plasma and respiratory tract lining fluid, serving as

an antioxidant pool in the immediate environment of a cell and for inter-organ transport of GSH (and Cys). Among the cell types known to export GSH are endothelial cells, neurons, fibroblasts, lymphocytes, mononuclear phagocytes, and type-I and -II pneumocytes present in the lower respiratory tract (Ballatori et al., 2009b). A sustained export of GSH has been measured, together with a lower though significant efflux of Cys, also in red blood cells (Giustarini et al., 2008).

Some members of the multi-drug resistance proteins (MRP), which, along with the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonylurea receptors (SUR), constitute the subfamily C of ATP-binding cassette (ABCC) proteins, act as cellular GSH efflux transporters (Fig. 1) as well as some members of the organic anion transporting polypeptide (OATP or SLC21A) family of transporters. The MRP mediate not only GSH efflux, but also export of GSSG and GSH-conjugates (Fig. 1) (Ballatori et al., 2009a; Franco and Cidlowski, 2012; Bachhawat et al., 2013). Although some carriers for the influx of GSH into the cytoplasm have been identified (such as Hgt1p in yeast (Bachhawat et al., 2013)), their presence in mammals is still discussed since the only report concerning the identification of this transporter in the rat turned out to be artefactual. Since CFTR and MRP2 are the only GSH carriers identified so far on the apical membrane of lung cells, these could play an important role in GSH delivery into the respiratory tract lining fluid, thus establishing the first line of defence against inhaled oxidants and noxious compounds.

GSH can be hydrolysed both in the cytoplasm and in the extracellular compartment. In humans and mouse, two ChaC-like proteins have been identified that are involved in the intracellular degradation of GSH, namely the ɣ-glutamylcyclotransferases ChaC1 and ChaC2. While ChaC2 expression and activity are constitutive and responsible for the continuous slow degradation of GSH (used primarily for Cys cellular supply), ChaC1 is upregulated during development and during stress conditions (Kumar et al., 2012; Kaur et al., 2017). The hydrolysis of GSH mediated by these enzymes leads to the formation of 5-oxoproline and cysteinylglycine, which are hydrolysed by a 5-oxoprolinase to produce glutamate and cleaved by cytosolic Cys-Gly peptidases to produce Cys and Gly, respectively (Fig. 1).

GSH is hydrolysed in the extracellular compartment on the surface of cells that express the ectoenzyme γ-glutamyl transpeptidase (GGT) (Fig. 1). The only enzymatically active proteins in this family are GGT1 and GGT5, which have a different localization and turnover rate (Hanigan et al., 2015). Under physiological conditions, the primary reaction catalysed by mammalian GGTs is the hydrolysis of γ-glutamyl amide bonds and, to date, they are the only enzymes that are known to initiate catabolism of GSH and GSH-containing molecules (*e.g.*, GSSG and GSH-conjugates). The transcriptional and translational levels of GGT synthesis, as well as its enzymatic activity, are redox-regulated (Hanigan et al., 2015). GGTs hydrolyse extracellular GSH releasing Glu and the dipeptide cysteinylglycine (CysGly) (Wickham et al., 2011). CysGly is readily oxidized to CysGly disulphide, or cystinylglycine ($(CysGlyS)_2$), in oxygenated extracellular compartment, such as the blood plasma (Rossi et al., 2009). CysGly is hydrolysed by the cell surface enzymes aminopeptidases (AP) or dipeptidases (DP) releasing Cys and Gly, which can then be transported into cells and used for GSH synthesis (Fig. 1). Specific amino acid transporters mediate cellular uptake of amino acids, whereas Pept2, a transporter of di- and tri-peptides (Frey et al., 2007), may mediate the absorption of CysGly.

The intra- and extracellular GSH concentrations are therefore the result of its anabolism and catabolism, as well as of its transport between the cytoplasm, the extracellular space, and the different organelles of the cell. Because of the differential tissue localization of transporters and GGT, there is an inter-organ directional flux of GSH in the body. Organs and tissues that present low GGT activity (*i.e.*, the liver and skeletal muscle) export GSH, which is carried by the plasma to cells having high GGT activity, which include the proximal tubular epithelial cells of the kidneys, small intestinal epithelial cells, and the Clara and type II epithelial cells of the lung (Zhang and Forman, 2009; Lash, 2011). The inter-organ GSH flux has the purpose to transport Cys in a nontoxic form between organs and tissues, maintaining high (mM) intracellular GSH concentrations where more necessary (Lu, 2009).

4. Glutathione function in the cellular antioxidant pathway

GSH is a key player of the redox regulative team of the cell (Trachootham et al., 2008). Even though the discovery of the essential role of GSH in iron-sulphur cluster biogenesis opens the possibility of its participation in iron metabolism (Kumar et al., 2011), its implication in the regulation of the cellular redox steady state is demonstrated by the presence of GSH-dependent antioxidant enzymes, by the fact that many enzymes of GSH metabolism are redox-regulated at transcriptional, translational, and kinetic levels, and by the fact that GSH acts as a signalling molecule to directly activate the expression of genes for cell redox regulation (Fratelli et al., 2005). Furthermore, a decrease in GSH levels contributes to oxidative stress, which has been associated with ageing and many pathological states, including neurodegeneration, inflammation, and infections (Ballatori et al., 2009b). Therefore, even if GSH can have pleiotropic functions in the different cellular compartments, in the cytoplasm it plays a critical role in the maintenance of the redox homeostasis and is involved both in the enzyme-catalysed detoxification reactions and in the direct conjugation with electrophilic aldehydes.

GSH alone can scavenge ROS, however in the cell it acts more efficiently (with higher rate constant) in concert with glutathione peroxidases (GPx) and glutathione reductase (GR) (Fig. 1) or with peroxiredoxin 6 (Prx 6) to reduce H_2O_2 into H_2O to limit its harmful effects (Fisher, 2011). The most predominant GPx is the GPx1, which is located in the cytoplasm of nearly all human cells and is found in most parenchymal organs and peripheral blood cells, particularly red blood cells (Lei et al. 2007). GPx catalyses the reduction of harmful peroxides using GSH as a reducing agent. These reductive reactions generate GSSG, which is potentially toxic to the cells. Cells must maintain adequate levels of GSH/GSSG ratio, and this can occur by either increasing the *de novo* intracellular synthesis of GSH or by reducing GSSG through the catalytic action of GR (Fig. 1). Moreover, when GSSG intracellular concentration exceeds the reductive capacity of the cell, as in conditions of severe oxidative stress, it is actively excreted from the cell by MRP (Fig. 1). Because of the action of the GR and MRP, the physiological GSH/GSSG ratio is ~100:1 in the cytosol. The GSH/GSSG ratio is crucial for normal cellular activities, and is involved in signalling processes, proliferation, differentiation, and apoptosis (Pallardó et al., 2009; Dalle-Donne et al., 2011). However, severe oxidative stress may overcome the ability of the cell to catalyse *de novo* GSH synthesis and/or the activity of GR may not be enough to maintain adequate cellular GSH/GSSG ratio, thus leading to accumulation of GSSG within the cytosol. Shifting the GSH/GSSG ratio toward the oxidizing state activates several signalling pathways, thereby increasing apoptosis and reducing cell proliferation (Wu et al., 2004). Moreover, the GSH/GSSG ratio controls the expression of several critical enzymes including GCL and GPx (Rahman et al., 2006). To protect the cell from a shift in the redox equilibrium, GSSG can also react with protein thiols (P-SH), leading to the formation of protein-glutathione mixed disulphides (*i.e.*, *S*-glutathionylated proteins, PSSG). *S*-glutathionylation is a post-translational modification that provides protection to P-SH from irreversible oxidation and/or participates in redox signalling (Dalle-Donne et al., 2009). *S*-glutathionylated proteins can be reversed to their GSH-free form by the enzyme glutaredoxin (Grx) (Fig. 1).

GSH is also important to protect cells and tissues from reactive aldehydes. The thiol group of GSH reacts with electrophilic aldehydes resulting in the formation of GSH-aldehyde adducts (GSH-conjugates). The conjugation of GSH with CS electrophiles can proceed sponta-

neously or, faster, by catalysis by glutathione-*S*-transferase (GST; Deponte, 2013, 2017; Mohana and Achary, 2017) (Fig. 1).

5. Effects of CS in human cell models

A wide range of bespoke or commercial set-ups, with different degree of sophistication, has been used to study the effects on the cells of exposure to tobacco smoke (Thorne and Adamson, 2013). A plethora of *in vitro* cell models has been developed alongside these exposure systems that may be used when assessing the cytological effects of CS. These cells can be either primary cell cultures of human origin or commercially available cell lines (Table 2).

Table 2
In vitro cell models used to assess the cytological effects of CS.

Model for	*In vitro* cells models	Exposure systems	Cigarette types	Analytical methods for determination of GSH and its disulphide forms	References
Oral epithelial tissue studies	HaCaT human keratinocyte cell line	Whole phase cigarette smoke (WCS). Acetaldehyde and acrolein	Commercial cigarettes (Dubek)	GSH were determined in cell lysates using the spectrophotometric method with DTNB. GSSG were determined after trapping GSH with 3-vinyl pyridine	Avezov et al., 2014
Oral connective tissue studies	HGF human gingival fibroblasts	Whole phase cigarette smoke (WCS)	3R4F research-reference cigarettes	GSH and its disulphide forms (GSSG and S-glutathionylated poteins) were measured with HPLC after derivatization with monobromobimane. GSH–acrolein and GSH–crotonaldehyde adduct were analyzed by LC–MS/MS	Colombo et al., 2012
Airway epithelium studies	BEAS-2B human bronchial epithelial cells	Cigarette smoke condensate (CSC)	3R4F research-reference cigarettes	GSH and GSSG were determined in cellular lysates by an enzymatic method using a commercial glutathione assay kit	Altraja et al., 2013
Airway epithelium studies	BEAS-2B human bronchial epithelial cells	Cigarette smoke extract (CSE)	3R4F research-reference cigarettes	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method	Benedikter et al., 2017
Airway epithelium studies	BEAS-2B human bronchial epithelial cells	Whole phase cigarette smoke (WCS). Vitrocell system	3R4F research-reference cigarettes	GSH and GSSG levels were determined in cell lysates using HPLC with fluorescence detection	Anthérieu et al., 2017
Airway epithelium studies	Calu-3 human bronchial epithelial cell lines	Gaseous phase of cigarette smoke	Unspecified	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method	Cantin et al., 2006
Airway epithelium studies	Human primary bronchial epithelial cells	Gaseous phase of cigarette smoke	2R4F research-reference cigarettes	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method	van der Toorn et al., 2007
Airway epithelium studies	16-HBE human bronchial epithelial cells	Cigarette smoke extract (CSE)	Commercial cigarettes (Marlboro Red)	GSH concentrations were evaluated using monochlorobimane by means of an inverted fluorescence microscope	Bazzini et al., 2013
Airway epithelium studies	16-HBE human bronchial epithelial cells	Cigarette smoke extract (CSE)	Commercial cigarettes (Marlboro Red)	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method	Pace et al., 2013
Airway epithelium studies	NCI-H292 human bronchial epithelial cells	Aqueous aerosol extracts	3R4F research-reference cigarettes	GSH/GSSG ratio was determined using the Promega GSH/GSSG-Glo Assay (luciferin-based assay)	Taylor et al., 2016
Airway epithelium studies	SAEC primary human small airway epithelial cells	Cigarette smoke extract (CSE)	1R3F research-reference cigarettes	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method	Kode et al., 2006
Airway epithelium studies	SAEC primary human small airway epithelial cells	Cigarette smoke extract (CSE)	2R4F research-reference cigarettes	GSH and GSSG were measured with HPLC after derivatization with monobromobimane.	Kariya et al., 2008
Airway epithelium studies	normal human primary bronchial epithelial cells	Whole mainstream smoke solutions (WSSs)	Commercial cigarettes (Marlboro Red and Marlboro Silver)	GSH/GSSG ratio was determined using the Promega GSH/GSSG-Glo Assay (luciferin-based assay)	Cao et al., 2017
Lung tissues studies	epithelial cells	Cigarette smoke extract (CSE)	1R3F research-reference cigarettes	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method	Kode et al., 2008
Lung tissues studies	A549 human type II alveolar epithelial cells	Cigarette smoke extract (CSE)	1R3F research-reference cigarettes	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method	Kode et al., 2006
Lung tissues studies	A549 human type II alveolar epithelial cells	Cigarette smoke condensate (CSC)	Commercial cigarettes (Gold Flak)	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method. GSSG were determined after trapping GSH with 3-vinyl pyridine	Kaushik et al., 2008
Lung tissues studies	A549 human type II alveolar epithelial cells	Cigarette smoke extract (CSE)	3R4F research-reference cigarettes	S-glutathionylated poteins were determined by DTNB	Kuipers et al., 2011
Lung tissues studies	Primary human lung fibroblast strains	Cigarette smoke extract (CSE)	1R3F research-reference cigarettes	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method. GSSG were determined after trapping GSH with 3-vinyl pyridine	Baglole et al., 2006
Lung tissues studies	HFL-1 primary human fetal lung fibroblasts	Cigarette smoke extract (CSE)	1R3F research-reference cigarettes	Total GSH was determined by the DTNB-GSSG/glutathione reductase recycling method	Baglole et al., 2008

Commonly, *in vitro* cell models are exposed to whole phase (mainstream) cigarette smoke (WCS, or smoking chamber model), to a specific fraction of CS (*i.e.*, CS extract (CSE), CS condensate (CSC) or gaseous phase), or any of CS individual components, such as saturated and unsaturated aldehydes (Dalle-Donne et al. 2016). While exposure to individual components makes it easier and clearer to identify the effects determined by the single components of the CS, exposure to WCS is needed to assess the additive effects of a complex mixture of molecules, which can be masked, different or completely absent using the individual components. The cigarettes used in many *in vitro* cellular models of exposure to CS are the Kentucky research-reference cigarettes (Table 2). The most popular research-reference cigarettes, *i.e.* the 3R4F ones, designed to be more representative of the most popular products on sale in the US cigarette market, are blended king-sized cigarettes with a cellulose acetate filter and an International Organization for Standardization (ISO) tar yield of 9.4 mg/cig in nine puffs (Roemer et al., 2012). The mainstream smoke chemistry yields from different types of Kentucky research-reference cigarettes have been published (Johnson et al., 2009; Eschner et al., 2011; Roemer et al., 2012; Schaller et al., 2016; Savareear et al., 2018).

One of the most common consequences of the exposure of cultured cells to CS is the induction of oxidative stress, leading to the generation of reactive oxygen species (ROS) that mediate the cell injury. In many *in vitro* cellular models of exposure to CS (*e.g.* ECV-304 cells, 16-HBE cells, NCI-H292 cells, and normal primary bronchial epithelial cells), intracellular ROS generation was measured with the probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which is internalized by the cells and converted to its fluorescent form 2′,7′-dichlorofluorescein by intracellular ROS (Gornati et al., 2013; Bazzini et al., 2013; Taylor et al., 2016; Cao et al., 2017). For example, in ECV-304 cells, exposure to CSE induced an increase in intracellular ROS generation, which was largely dose-dependent (Fig. 2). Another consequence of the CS-induced oxidative stress is lipid peroxidation, which leads to the formation of α,β-unsaturated aldehydes such as malondialdehyde and 4-hydroxy-2-nonenal (4-HNE). Malondialdehyde has the power to inactivate many cellular proteins by generating protein cross-linkages (Jacobs and Marnett, 2010). Increased levels of malondialdehyde have been measured in human lung epithelial cells (A549) and human bronchial epithelial cells (BEAS-2B) exposed to both CSE and WCS (Zhang et al., 2017; Somborac-Bačura et al., 2018) 4-HNE has many cytotoxic effects: it induces cytoplasmic Ca^{2+} accumulation, stimulates expression of proinflammatory cytokines and NF-κB, leads to mitochondrial dysfunction and apoptosis (Breitzig et al., 2016). Increased production of 4-HNE has been measured in several *in vitro* cellular models exposed to CSE, including primary human lung fibroblasts (Baglole et al., 2006), human HaCaT keratinocyte cell line (Sticozzi et al., 2012), and human alveolar epithelial type I like (ATI-like) cells (Kosmider et al., 2011) (Fig. 3).

Other consequences of exposure to CS observed in *in vitro* cell models are reversible and irreversible protein modifications. Among reversible modifications that can be involved in redox regulation of protein functions (Dalle-Donne et al., 2011), both protein *S*-nitrosylation (Sathish et al., 2015) and *S*-glutathionylation (Kuipers et al., 2011) have been observed. Among irreversible modifications, protein carbonylation may contribute to cellular dysfunction and disease pathogenesis (Dalle-Donne et al., 2006) and has been widely studied (Dalle-Donne et al., 2017). Moreover, CS also induces oxidative damage to nucleic acids (*e.g.*, Deslee et al., 2010; Cao et al., 2016). Of the bases in both DNA and RNA, guanine is the most susceptible to oxidation and has been the most commonly studied marker of nucleic-acid oxidation. ROS can oxidize guanine to produce 8-oxo-7,8-dihydro-2′-deoxyguanosine (8OHdG) in DNA, which is considered as reliable biomarker for oxidative DNA damage and 8-oxo-7,8-dihydroguanosine (8OHG) in RNA (Halliwell and Gutteridge, 2007). A positive correlation exists between CS and oxidative DNA damage response. For example, CS-induced DNA oxidation was observed in bronchial epithelial cells *in vitro* and *in vivo*. In human samples, the expression of 8-OHdG was significant higher in smokers than non-smokers both in lung cancer patients and non-cancer controls (Cao et al., 2016). Nucleic-acid oxidation was demonstrated in alveolar lung fibroblasts of patients with severe emphysema, which was associated with changes in the expression of genes involved in the repair of oxidized nucleic acids (Deslee et al., 2010). In the mouse model of CS-induced emphysema, a time-dependent accumulation of oxidized DNA and RNA was shown in alve-

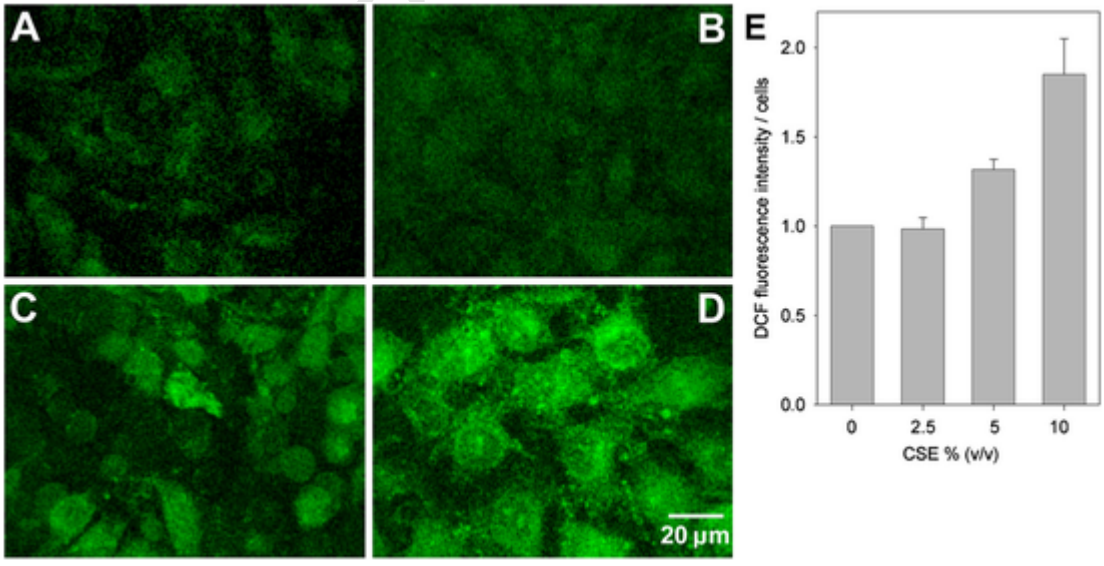

Fig. 2. Intracellular ROS formation after exposure of ECV-304 cells to CSE. (A-D) Representative microphotographs of three independent experiments showing the increase in dichlorofluorescein fluorescence induced by exposure of ECV- 304 cells to 0% (A), 2.5% (B), 5% (C), and 10% (D) CSE. (E) Bar-graph shows quantitative data of dichlorofluorescein fluorescence per single cell compared to that of control cells. It is worth noting that the ECV-304 cell line was for long used as a model for human endothelium because it was originally believed to be a spontaneously transformed human normal endothelial cell line. Actually, it is derived from the human bladder carcinoma T24 cell line (American Type Culture Collection Standards Development Organization Workgroup ASN-0002, 2010). Reprinted with permission of Elsevier from (Gornati et al., 2013) with slight modifications. To see this illustration in colour, the reader is referred to the web version of this article.

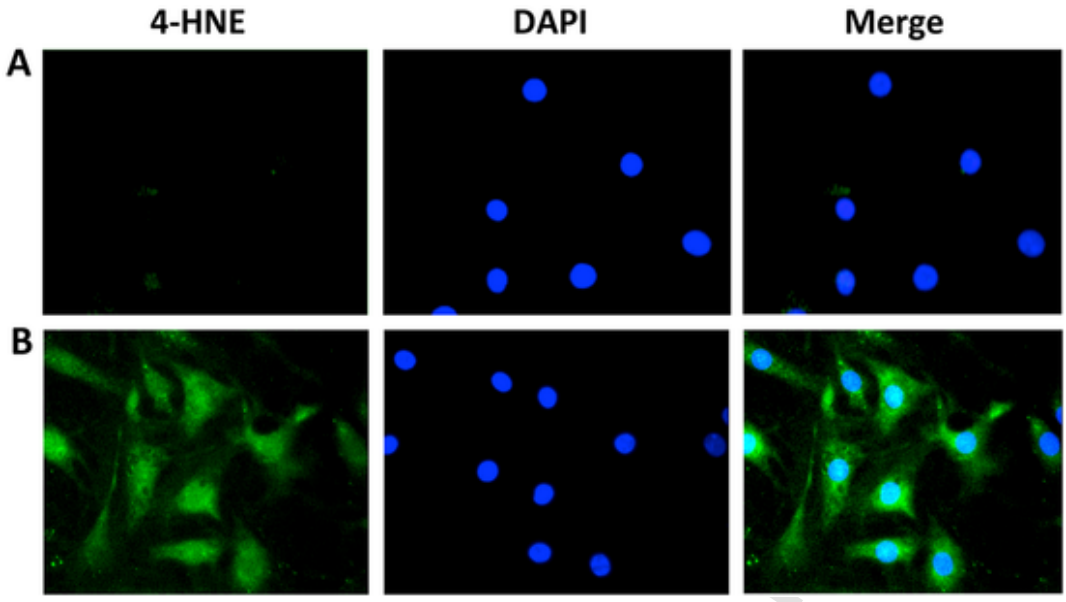

Fig. 3. CSE induces production of 4-HNE in human alveolar epithelial type I like (ATI-like) cells. (A) Control. (B) ATI-like cells were exposed to 5% CSE for 24 h. Cells were probed by 4-HNE primary antibody, stained with Alexa Fluor 488 as secondary antibody (green) and mounted with Vectashield medium containing DAPI, 4′,6-diamidino-2-phenylindole (blue). Representative microphotographs from three independent experiments are shown. Reprinted from (Kosmider et al., 2011) with slight modifications. To see this illustration in colour, the reader is referred to the web version of this article. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

olar fibroblasts, associated with an increase in mRNA expression of genes involved in the repair of oxidized nucleic acids. *In vitro* studies of lung fibroblasts demonstrated that CSE induces ROS-dependent nucleic oxidation (Deslee et al., 2010). In addition, the prevalence of nucleic-acid oxidation in alveolar wall cells correlates with the severity of human emphysema (Deslee et al., 2009).

Given the multiple role of GSH in cell pathophysiology, we think it is worthwhile to have the first overview of CS-induced changes to GSH levels in *in vitro* cell models. From here to follow, we will explore and give a synthesis of the literature, dividing the results according to the different cytotypes.

5.1. Effects of CS on GSH in cell models of the human oral cavity

The cells that make up the tissues of the oral cavity are the first to encounter inhaled CS and are uniquely susceptible to oxidative damage (Avezov et al., 2015), because the oral mucous membranes allow rapid absorption of toxic constituents of CS across their surface. The human keratinocyte cell line HaCaT is widely used as a model for epithelial tissue studies, including oral epithelium investigations. Avezov and colleagues demonstrated that a single puff of WCS caused a decrease in GSH levels to 34% of the initial content, while two and three puffs almost erased GSH activity compared to non-exposed cells. Similar decrease in GSH levels was obtained after exposure of HaCaT cells to acrolein (Avezov et al., 2014). Acrolein reactivity is due to its double bond, which reacts with thiol groups (Kehrer and Biswal, 2000). Therefore, acrolein can react with the thiol group of GSH, thus diminishing its availability for the antioxidant function. Otherwise, exposure of HaCaT cells to acetaldehyde, which has no reactive double bond, left GSH levels similar to those of control cells, and no parallel increase in GSSG level was observed (Avezov et al., 2014).

The effects of exposure to CS on the gingival epithelium have been investigated in human gingival epithelial organotypic cultures, in which normal gingival keratinocytes form a fully differentiated three-dimensional epithelial tissue (Fig. 4). Human gingival epithelial organotypic cultures show several effects of *in vivo* exposure to CS, including the release of inflammatory mediators and activation of cell stress networks (Schlage et al., 2014). A recent study examined the effects on GSH after exposure of organotypic gingival cultures to CS (Zanetti et al., 2017). This study showed that, 4 h after exposure to CS, GSH levels were significantly diminished. Moreover, several metabolites of the GSH cell pathways were altered by exposure to CS, including a depletion of Cys and Gly, increases in γ-glutamyl amino acids, 5-oxoproline, *S*-adenosyl homocysteine and 2-hydroxybutyrate, and a decrease in *S*-adenosyl methionine and serine (Zanetti et al., 2017).

The epithelial cells, acting as mechanical barrier, can reduce but not eliminate completely the injurious effect of the smoke components on gingival and periodontal fibroblasts (Giannopoulou et al., 2001); therefore, it sounds appropriate to include here studies of CS effects on human gingival fibroblasts (HGF) (Colombo et al., 2012). After HGF exposure to 0.5–12 puffs of WCS, total glutathione (GSH + 2GSSG + PSSG) decreased dramatically, indicating that intracellular GSH depletion could be a mechanism for WCS-induced cytotoxicity. WCS-induced depletion of intracellular GSH was not accompanied by a corresponding formation of GSSG and PSSG. In particular, HGF exposed to 12 puffs exhibited the largest decline in GSH level, which was only minimally compensated for by oxidation of GSH to GSSG and by little increases in PSSG levels (Fig. 5). Intracellular GSH decrease was mainly due to the export of GSH-acrolein and GSH-crotonaldehyde adducts (Fig. 6) (Colombo et al., 2012). Findings in HGF thus reveal that ROS and aldehydes are, at least partly, responsible for the CS-induced intracellular decrease in GSH concentration because of their covalent conjugation with GSH, resulting in decreased antioxidant defences in oral connective tissues.

GSH depletion caused by exposure to CS makes it unavailable for the enzymatic reducing cycle system, which is normally activated after oxidative stress occurrence and the formation of GSSG and/or PSSG. Exhaustion of GSH could induce a chronic lack of antioxidant protection in the cells of the oral cavity. In that case, chronic smokers might inhale more CS-related ROS and aldehydes than those that could be scavenged by the residual antioxidants, resulting in increased vulnerability to oxidative stress of oral cavity tissues.

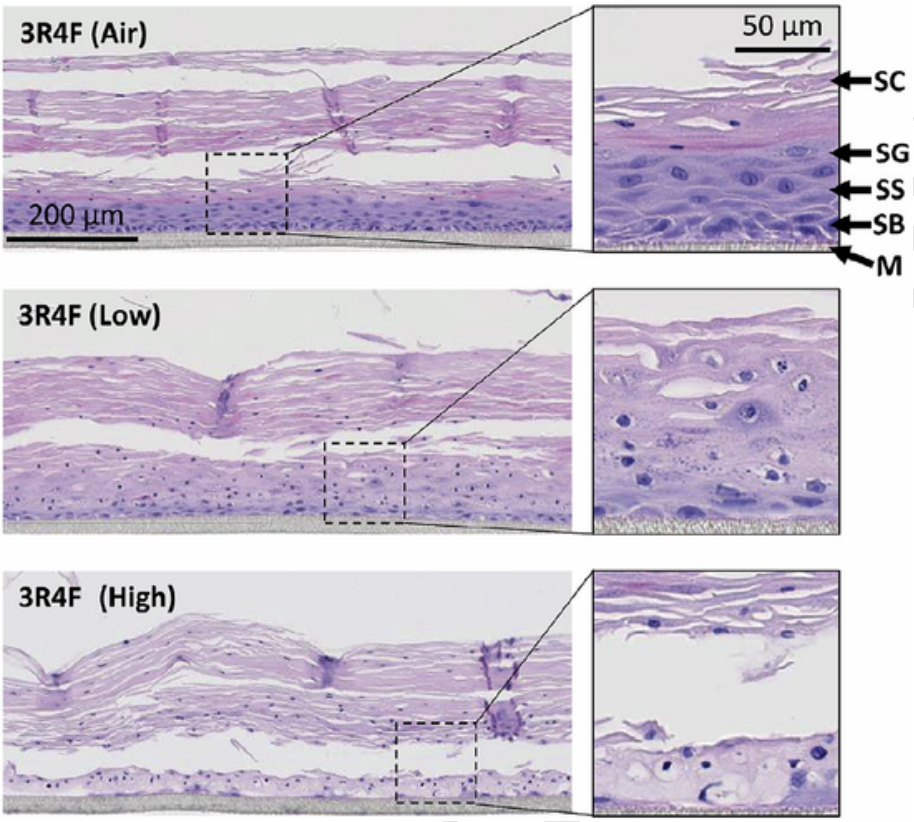

Fig. 4. Effects of exposure to CS on human gingival epithelial organotypic cultures. Representative microphotographs of hematossilin-eosin stained gingival cultures after 24 h from the last exposure to CS (3R4F research-reference cigarettes). Abbreviations indicate different layers of gingival cultures: M, membrane; SB, stratum basale, SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum. Images show 20× magnification and 63× magnification for image insets. Reprinted from (Zanetti et al., 2017) with slight modifications. To see this illustration in colour, the reader is referred to the web version of this article.

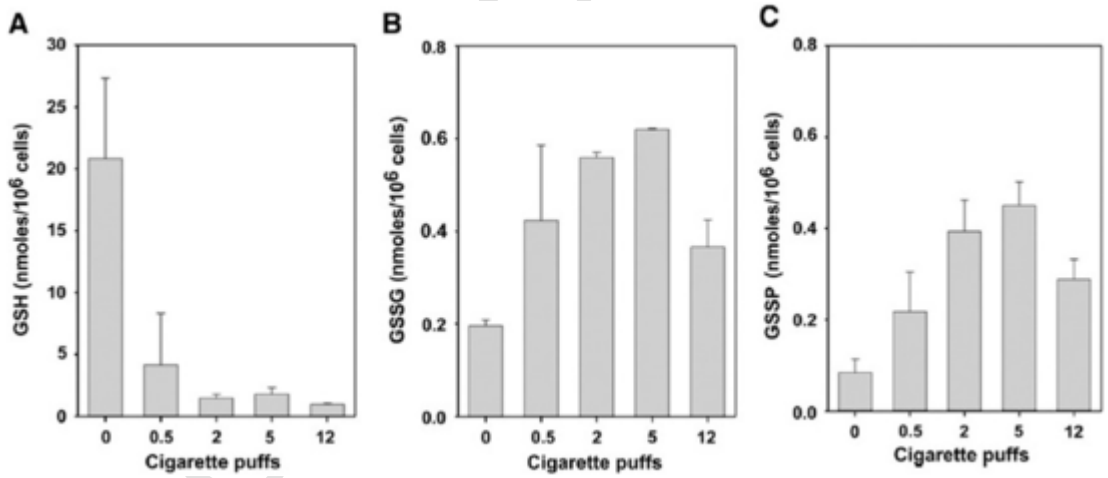

Fig. 5. Levels of GSH, GSSG and *S*-glutathionylated proteins in human gingival fibroblasts (HGF) exposed to CS. Changes in (A) GSH and (B) GSSG levels in whole-cell lysates from HGF exposed to 0, 0.5, 2, 5, and 12 cigarette puffs (3R4F research-reference cigarettes). (C) Protein *S*-glutathionylation in whole-cell lysates from HGF exposed to 0, 0.5, 2, 5, and 12 cigarette puffs. Results are expressed as the means ± SD of three replicate measurements. Reprinted with permission of Elsevier from (Colombo et al., 2012) with slight modifications.

5.2. Effect of CS on GSH in cell models of the human respiratory epithelium

The airway epithelium is an important cellular barrier between the external epithelial lining fluid and the lung parenchyma. Therefore, the airway epithelial cells are immediately and directly exposed to any change in the redox environment on the airway surface, which makes them especially susceptible to CS-induced oxidative damage.

Exposure of human primary bronchial epithelial cells to the gaseous phase of CS for 5 min decreased free thiols and irreversibly modified a substantial amount of total glutathione (GSH + GSSG) to GSH-acrolein and GSH-crotonaldehyde adducts (van der Toorn et al., 2007). Therefore, CS does not oxidize GSH to GSSG but, rather, reacts with aldehydes to form non-reducible GSH-aldehyde adducts, thereby depleting the total intracellular GSH pool, preventing the activation of the enzymatic redox cycle and the formation of GSSG and/or PSSG. Deple-

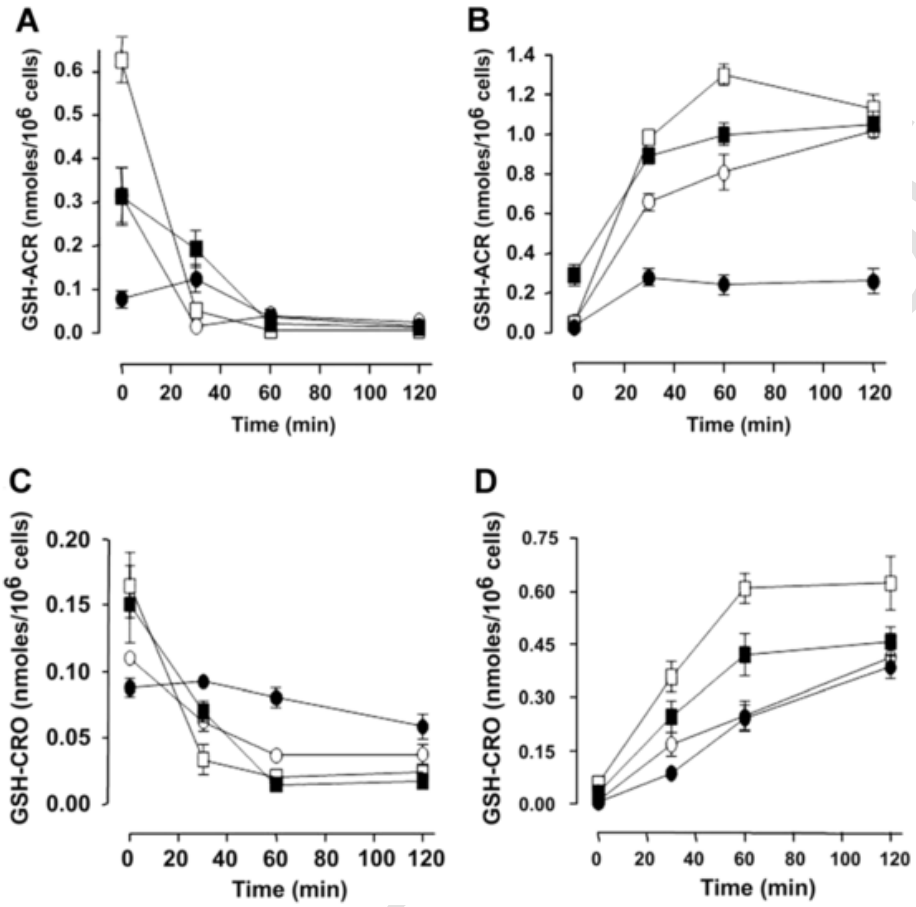

Fig. 6. Liquid Chromatography-Electrospray Ionization-tandem Mass Spectrometry (*LC-ESI-MS/MS*) quantitative analysis of intracellular and extracellular GSH-aldehyde adducts in human gingival fibroblasts (HGF) exposed to CS. LC–ESI–MS/MS quantitative analysis of (A) intracellular and (B) extracellular GSH-acrolein adducts after HGF exposure to 0.5 (●), 2 (○), 5 (■), and 12 (□) cigarette puffs. LC–ESI–MS/MS quantitative analysis of (C) intracellular and (D) extracellular GSH-crotonaldehyde adducts after HGF exposure to 0.5 (●), 2 (○), 5 (■), and 12 (□) cigarette puffs. Values are expressed as data ± SD of three independent experiments (duplicate injection) ($n = 3$). All data were statistically different (*versus* time 0) except for those relative to 0.5 cigarette puff (at least $p < .05$). Reprinted from (Colombo et al., 2012) with slight modifications.

tion of the intracellular GSH pool could induce a chronic lack of antioxidant protection in the cells of the respiratory epithelium.

Exposure of human bronchial epithelial BEAS-2B cells to CSC caused a significant initial decrease in intracellular GSH concentration at 1 h compared to non-exposed cells (Altraja et al., 2013). Thereafter, the intracellular GSH concentration returned to a level not different from that of the control at 3–6 h and then increased without any significant change in GSSG concentration. The increase in GSH synthesis was related to an increase in GCL subunit transcription: exposure to CSC increased mRNA expression of both catalytic (GCLC) and modulatory (GCLM) subunits of GCL (Altraja et al., 2013), with a trend in the increase in GCLM expression that mimicked in all that observed for GSH, unlike what was observed for GCLC. This led the authors to conclude that an increase in GCLM expression is essential for the regulatory GSH increase observed after exposure to CSC (Altraja et al., 2013). These findings suggest the possibility that, in the large airway epithelial cells, the GCLM subunit is more influential than GCLC in maintaining the GSH homeostasis during cigarette smoking. A rebound in GSH synthesis, in parallel to a slight increase in GCLC and stronger augment in GCLM mRNA expression, has been described also in bronchial epithelial 16-HBE cells, an SV40-immortalized human *bronchial epithelial* cell line, after a 24-h exposure to CSE (Bazzini et al., 2013). In 16-HBE cells, also the mRNA of GR, which regenerates GSH from GSSG, is increased both after 3 h and 24 h of exposure to CSE. The mRNA expression of GPx2 and 3 was increased in a similar way, while that of GPx1 mRNA, which appeared to be unaffected after 3 h, significantly decreased after 24 h. The increase in GPx3 expression and secretion in the lung could help in counteracting the ROS burden in the epithelial lining fluid (ELF). These results are well in agreement with those of another study performed in 16-HBE cells and showing that total intracellular glutathione is not affected after 18-h exposure to CSE (Pace et al., 2013).

An adaptive increase in cellular GSH content has been demonstrated during chronic exposure to smoke of primary human small airway epithelial cells and normal (C38) or CFTR deficient (IB3) bronchial epithelial cells (Kode et al., 2006; Kariya et al., 2008). The latter study showed that the increase in intracellular GSH was accompanied by an increase in GSH secretion, with a consequent increase in the GSH concentration in the extracellular medium. ELF is the first barrier between the lung and oxidants produced by CS; therefore, the airways should be able to keep high levels of antioxidants in the ELF as part of an adaptive mechanism. The increase in the concentration of extracellular GSH was also observed, though to a lesser extent, in CFTR-deficient (IB3) cells exposed to CSE, suggesting that, in addition to the CFTR channel, other GSH transporter(s) were responsible of the efflux of GSH.

Exposure of epithelial Calu-3 cells, a human non-small-cell lung cancer cell line that grows in adherent culture and displays epithelial morphology, to CS for 24 h was associated with increases in cellular GSH levels and GCLC expression and decreased CFTR expression at the gene, protein, and functional levels. However, the extracellular concentration of GSH was not measured (Cantin et al., 2006). BEAS-2B cells

exposed to CSE for 8 days showed a marked reduction of intracellular GSH concentration and GCLC mRNA expression (Cheng et al., 2016). The latter was attributed to a specific hypermethylation of the GCLC promoter, which was not seen on the promoters for genes of other antioxidant enzymes, thus demonstrating a specific epigenetic regulation of the GCLC gene by CS.

The effects of exposure to CS on GSH have also been studied in *in vitro* human air-liquid-interface (ALI) airway tissue models, which allow obtaining cells with morphological and physiological characteristics more similar to those of the airways (Talikka et al., 2014). The structural similarity between the *in vitro* human ALI airway tissue models and the epithelial lining of human airways allowed measuring CS-induced cytotoxicity, the integrity of the tissue barrier, the matrix metalloproteinase secretion, and the mucus over-accumulation on the apical surface of the ALI cultures, possibly due to goblet cell hyperplasia and cilia paralysis (Haswell et al., 2010). To investigate the effect of the whole mainstream smoke solution (WSS), which contains particulate and gas phase constituents of mainstream CS, on intracellular GSH in normal human primary bronchial epithelial cells cultured at the ALI, the intracellular GSH content of these cells was assessed at different times after exposure to WSS generated by smoking two different types of commercial cigarettes by means of a smoking machine. A single 4-h treatment with WSS resulted in a marked decrease in the GSH/GSSG ratio at all the CS doses tested. No data are reported for prolonged exposure (Cao et al., 2017).

In BEAS-2B cells cultured at the ALI, the GSSG/GSH ratio increased immediately after 8-min and 48-min exposures to CS (Anthérieu et al., 2017). However, the increase was transient and no significant increase in the GSSG/GSH ratio was measured 3 h after the end of exposure, suggesting that BEAS-2B cells cultured at ALI elicit an adaptive response (Anthérieu et al., 2017). However, a limit of this study is that it does not report absolute values, but only percentage changes compared to the control.

The studies reported above suggest that epithelial cells in large and small airways respond similarly to exposure to CS relative to intracellular GSH levels. Short-term (acute) exposures (in most cases 5 to 60 min) to CS caused a significant depletion of intracellular GSH levels. Conversely, long-term (chronic) exposures (in most cases 3 to 24 h) to CS were ineffective on GSH intracellular levels. These results could be explained as an adaptive response to a long-term exposure to CS. In effect, a prolonged exposure to CS induces a GSH increase due to upregulation of the expression and activity of the enzymes involved in GSH synthesis (Cantin et al., 2006; Altraja et al., 2013; Bazzini et al., 2013).

In human alveolar epithelial type I like (ATI-like) cells, exposure to CSE led to lipid peroxidation and the formation of 4-HNE (Kosmider et al., 2011). Immunostaining of ATI-like cells with primary antibody against 4-HNE showed an intense staining in the cytoplasm and nucleus, suggesting that CSE increases 4-HNE production as a consequence of oxidative stress (Kosmider et al., 2011). In transformed human alveolar type II epithelium-like A549 cells, a depletion of intracellular GSH concentration after short-term (5 min) exposures to CS was demonstrated (van der Toorn et al., 2007). In particular, similar to what the authors also observed in bronchial cells, exposure of A549 cells to gaseous phase of CS decreased free thiols and irreversibly modified a substantial amount of total glutathione (GSH + GSSG). Mass spectrometry analysis showed that GSH was modified to GSH-acrolein and GSH-crotonaldehyde adducts (van der Toorn et al., 2007).

In lung A549 cells, differently from what had been observed in primary human small airway epithelial cells, the long-term (24-h) exposure to various concentrations of CSE, which collects the aqueous phase of gas/vapour phase of CS and, therefore, contains most of the components inhaled by smokers, caused a depletion of GSH and a decrease of GCL activity and GCLC expression (Kode et al., 2008). Prolonged exposure of A549 cells to various concentrations of CSE for up to 48 h resulted in diminished biosynthesis of GSH in a concentration-dependent manner (Radan et al., 2019). The highest concentrations of CSE also inhibited GCL mRNA expression (Radan et al., 2019). Conversely, after long term exposure of A549 cells to CSC, which collects the lipid-soluble phase of CS, intracellular GSH and GSSG levels increased proportionally to the increase in the concentration of CSC (0.1, 10 and 50 μg/ml) (Kaushik et al., 2008). Maximum increase in GSH and GSSG levels was induced by 50 μg/ml of CSC, whereas GSH concentration was drastically decreased by CSC concentrations higher than 50 μg/ml (Kaushik et al., 2008). Moreover, CSC decreased GSH/GSSG ratio in a concentration dependent manner, with a maximum effect at 50 μg/ml. Exposure of A549 cells to CSC for 24 h also increased dose-dependently GCL activity and GCL mRNA levels (Kaushik et al., 2008). Maximum transcriptional activation and synthesis of mRNA was observed at 50 μg/ml of CSC.

Another study showed that, in A549 cells, CSE induces a decrease in glutaredoxin 1 (Grx1) mRNA and protein expression, in addition to decreased Grx1 activity and increased protein *S*-glutathionylation, suggesting a role for the Grx1/*S*-glutathionylation redox system in CS-induced lung epithelial cell death (Kuipers et al., 2011).

In conclusion, these studies show that, even in alveolar epithelial cells, similar to what observed in the airway epithelium, exposure to CSE induces an initial reduction in the concentration of GSH. This is followed, in the long run, by an adaptive response in which, in parallel to an increase in the activity of enzymes involved in the GSH pathway, an increase in the concentration of GSH occurs. Too high concentrations of CSE may prevent activation of this adaptive response.

5.3. Effect of CS on GSH in human lung fibroblasts

Lung fibroblasts, whose primary function is the production of extracellular matrix for tissue maintenance and repair, are targets of the water-soluble components of CS that pass through the basement membrane (Ishii et al., 2001). In primary human lung fibroblast strains exposed to CSE for 3, 6, and 24 h, CSE increased 4-HNE levels in the cytoplasm, nucleus, and in nuclear membrane, as shown by immunocytochemical staining with an antibody against 4-HNE (Baglole et al., 2006). All strains of lung fibroblasts exhibited an initial decrease in intracellular GSH levels when exposed to CSE for 3 h without concurrent increase in GSSG concentration (Baglole et al., 2006). However, when the cells were exposed to CSE for 24 h, GSH returned to near-baseline levels of untreated cells in some but not all fibroblast strains (Baglole et al., 2006). These findings suggest that lung fibroblasts from different individuals vary in their sensitivity to CSE and have intrinsically diverse capabilities to recover from exposure to CS. In addition, primary human foetal lung fibroblasts exposed to CSE showed increased expression of the cytoprotective enzyme inducible haeme oxygenase (HO)-1, which was not induced by Nrf2 and correlated to a decrease in intracellular GSH concentration (Baglole et al., 2008). These findings suggest that HO-1 upregulation may play a protective role in lung fibroblasts to counteract oxidative stress caused by CS. Similarly, CS induces HO-1 expression also in alveolar epithelial cells and in alveolar macrophages (Fukano et al., 2006; Slebos et al., 2007).

6. Conclusion and perspectives

Although reference cigarettes are often used in the various cell models of exposure to CS, the various "types" of CS used, *i.e.*, WCS, CSE and CSC, have important differences, especially in relation to the water-soluble substances, that depend on how they are prepared. Furthermore, as with other xenobiotics, the use of the nominal concentration of CSE as the only measure of cell exposure is inadequate (Bourgeois et al., 2016). In fact, the cell response to CSE is more closely related to the amount of bioavailable chemicals per cell and, therefore, depends on

the nominal concentration of CSE, the total number of cells, and the volume of CSE solution used. Hence, the results of different *in vitro* cellular models cannot simply be compared to each other. Moreover, one must be cautious when trying to extrapolate the *in vitro* findings to smokers, because *in vitro* cell models of exposure to CS suffer many limitations, discussed below.

In vivo cells are not exposed directly to CS, but rather to the components of CS that have been dissolved into extracellular fluids: *e.g.*, saliva covering the oral epithelium and respiratory tract lining fluid covering the respiratory epithelium. Additionally, *in vitro* studies mainly use aqueous CSE (Table 2), whereas smokers inhale CS directly. Approaches that evaluate specific components of CS (*e.g.*, saturated and α,β-unsaturated aldehydes) are also problematic. Although the effects on GSH of individual components of CS can be assessed, it is likely that the cell response to complex mixtures such as CS is not the sum of multiple independent molecules.

Some *in vitro* studies are conducted with transformed cells lines (Table 2) and, therefore, might not reflect normal cell behaviour. Cells that are transformed to proliferate indefinitely often show variations in their signalling pathways. Additionally, a cell line may have acquired mutations with shifting of the genotype and phenotype.

Differently from cultured cells, our body's cells are not normally exposed to antibiotics (or antimycotic agents) frequently added to the culture medium. The endocrine environment is also very different between *in vitro* cell models and cells in their physiological environment. Although suitable constituents are present into the culture medium, their concentrations may be different from those found in extracellular fluids *in vivo*. Moreover, the mechanical forces caused by the flow of body fluids are also totally absent in cell cultures.

Cultured cells are in a state of hyperoxia (Halliwell, 2011). Except for the respiratory epithelium, our body's cells are exposed to O_2 partial pressure (pO_2) in the range of ~10–100 mmHg (*i.e.* ~1.1–13%) (Sullivan et al., 2006; Carreau et al., 2011), but *in vitro* cell cultures are mostly performed in 95% air (79% N_2/21% O_2) and 5% CO_2, thus providing about 150 mmHg (*i.e.* ~19.9%) O_2. Rates of production of ROS by leakage from electron transport chains (particularly in the mitochondria) or by cellular enzymes (*e.g.* xanthine oxidase) appear to be O_2-limited at 10 mmHg. Therefore, ROS levels increase if O_2 levels are raised (Halliwell, 2011). This entails that cultured cells are under oxidative stress (Halliwell, 2003; Halliwell and Gutteridge, 2007; Halliwell, 2014).

In vitro cell models are, sometimes, in a state of hyperglycaemia (Halliwell, 2014). *In vivo*, human cells are normally exposed to 4–5 mM glucose. Otherwise, the concentrations of glucose in culture media are variable: several media use 5.5 mM glucose, whereas other media use 11–55 mM glucose. High glucose concentrations for prolonged periods increase mitochondrial superoxide anion generation, leading to severe alterations of metabolism and cellular functions (Giacco and Brownlee, 2010; Crespo et al., 2010).

The quantification of GSH and its disulphide forms is problematic in cultured cells for several reasons (Giustarini et al., 2015, 2016). Firstly, basal levels of cellular GSSG and PSSG are very low compared to GSH levels; in addition, GSH easily undergoes auto-oxidation during sample preparation. Therefore, the auto-oxidation of a small amount of GSH to GSSG can dramatically alter the GSH/GSSG ratio and, consequently, the level of PSSG. Even if you routinely use several alkylating agents to bind GSH and prevent its oxidation, not all of them are equally effective. Therefore, you should pay special attention to choosing the most appropriate procedure for pre-processing samples. Secondly, GSSG and PSSG can be enzyme-reduced back to thiols during cell manipulations (Giustarini et al., 2015, 2016). Thirdly, quantification of GSSG and PSSG levels may be more complex in *in vitro* cell models than in other biological systems because different experimental conditions can be used to treat and collect them, thus introducing interfering factors (Giustarini et al., 2015, 2016). The use of the GSH-recycling method is not easy and requires some particular advices (Giustarini et al., 2013). In this regard, we have drawn up detailed protocols for the artefact-free and accurate measurement of GSH, GSSG, and PSSG in blood, solid tissues, and several cultured cell lines (Giustarini et al., 2013, 2015). We strongly support a critical approach to the analytical aspect, emphasizing the importance of paying attention to data obtained from untreated samples, which should be as much as possible homogeneous among different research groups.

It is also appropriate to consider that different results have been obtained in different *in vitro* cell models exposed to different conditions of CS. For example, a study on bronchial epithelial 16-HBE cells showed that *CFTR* gene expression is increased after a 48-h exposure to CS, suggesting that CFTR might be initially decreased, but, as the cell upregulates antioxidant adaptive responses, CFTR expression is induced as well (Gould et al., 2012). Differently, previous studies in bronchial epithelial Calu-3 cells exposed to gaseous phase of CS for 24 h suggested that CS decreased CFTR expression at the gene, protein, and functional levels, and increased cellular GSH (Cantin et al., 2006). Importantly, researchers demonstrated that CFTR deficiency also occurs in the nasal respiratory epithelium of smokers (Cantin et al., 2006).

Therefore, owing to the complexity of the effects of CS in whole-organ, results from *in vitro* studies should always be viewed with caution when trying to extrapolate to smokers. Because *in vitro* cell models usually lack complex interactions with other cell types as in whole-organ, the findings of *in vitro* cell models may not always reflect the responses in smokers.

Nevertheless, some studies have shown a substantial congruence between *in vitro* cell models and pathological changes occurring in smokers. For instance, studies in human bronchial epithelial 16-HBE and BEAS-2B cells and in healthy smokers highlighted up-regulation of GCL after long-term exposure (chronic) to CS. In 16-HBE cells, a prolonged exposure to CS induced GSH increase due to upregulation of GCLM expression and, consequently, a boost of GSH synthesis (Bazzini et al., 2013). In BEAS-2B cells, a prolonged exposure to CS induced increased mRNA expression of both GCLC and GCLM (Altraja et al., 2013). Assessment of GSH related gene expression by microarray analysis in small airway epithelium and alveolar macrophages of healthy smokers (n = 30, age 43 ± 1 years) compared with healthy non-smokers (n = 19, age 42 ± 2 years) showed increased gene expression of GCLC in small airway epithelium and alveolar macrophages of healthy smokers (Carolan et al., 2009). Results from humans and cellular models exposed to CS highlighted that, in epithelial cells of the airway epithelium, increase in GCL expression is essential for the increase in GSH synthesis. This finding is particularly interesting considering that smokers have a high GSH content in the ELF of the bronchi and the lung alveolar surfaces (Neurohr et al., 2003; Drost et al., 2005). Studies conducted in mice exposed to CS that assessed changes in the GSH level occurring in the ELF suggested that GCL activity may be responsible for the high ELF GSH content in smokers (Gould et al., 2010, 2011).

From what has been discussed above, it appears that, despite the many limitations, studies using *in vitro* cellular models have provided some useful insights. Therefore, the use of *in vitro* cellular models has some positive aspects. Firstly, for ethical reasons it is not always possible to obtain specimens from humans (smokers and non-smokers); therefore, the design of *in vitro* cellular models of exposure to CS seems to be ethically justified. Secondly, *in vitro* cellular models are more feasible because a single cell line can be studied more readily than in smokers. Moreover, in *in vitro* studies of exposure to CS many confounding factors (*e.g.* age, sex, comorbidities, use of medications) that can alter the biological response of *in vivo* cells to CS are absent, whereas they need to be considered in human studies. Thirdly, *in vitro* cellular models are cheaper and more reproducible than the more complex *in vivo* systems. Lastly, cell culture studies can

be done under well-controlled conditions with simple adjustments to the dose and time of exposure to CS. About this, the different results that have been obtained in different *in vitro* cellular models exposed to CS can be mainly due to the different methods used to generate CS. However, recent technological advancement can help solve the difficult standardization of exposure to CS of cell cultures. Cigarette smoking machines are able to generate CS with a reproducible dose adjustment, making comparisons between different laboratories feasible (Thorne and Adamson, 2013; Adamson et al., 2014; Li, 2016). Finally, organotypic tissue cultures (Schlage et al., 2014; Iskandar et al., 2015; Zanetti et al., 2017) and *in vitro* ALI airway tissue models (Talikka et al., 2014; Li, 2016) are very promising and more advanced *in vitro* models for the study of CS-related effects on smokers. In addition and no less important, they are a promising alternative to animal experimentation.

In conclusion, only studies in smokers will serve as definitive tests for the assessment of CS-induced damage and the consequent changes in GSH levels. However, *in vitro* cellular models remain important tools in assessing CS-related damage. A cautious interpretation of experimental results, recognizing limitations of the specific *in vitro* cell models used, is essential if we want to move forward with the understanding of damage due to the habit of smoking.

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Declaration of Competing Interest

The authors declare that they have no known conflicts of interest associated with this publication. We further confirm that the manuscript has been read and approved by all named authors and that the order of authors listed in the manuscript has been approved by all of us.

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