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Effect of tobacco and nicotine replacement therapy on biomarkers associated with endothelial dysfunction

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in Biomedical and Pharmacological Sciences

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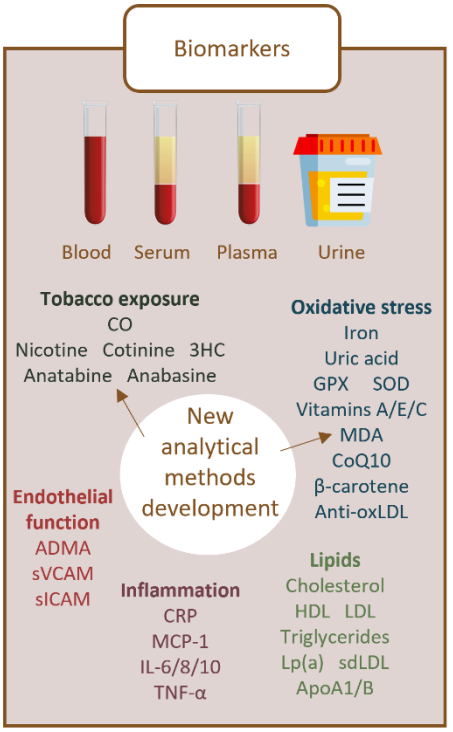
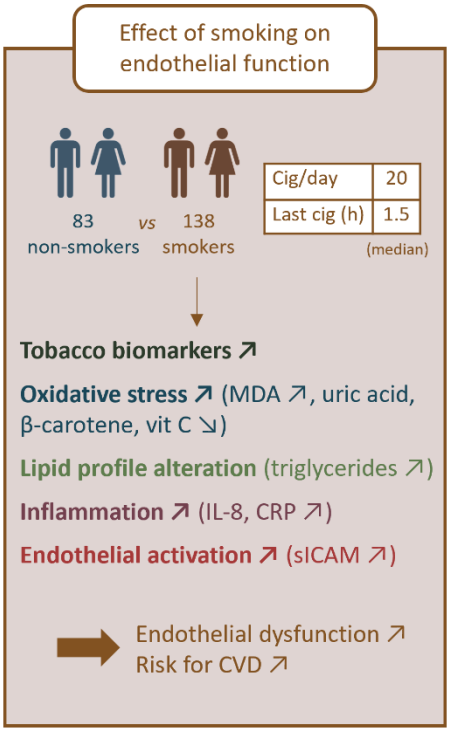
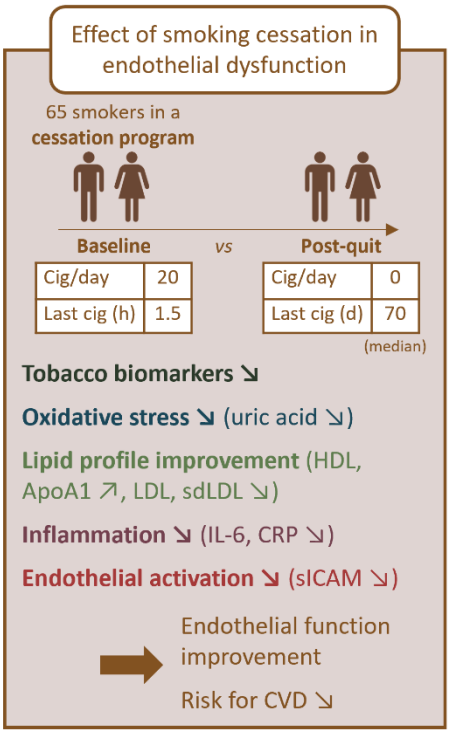
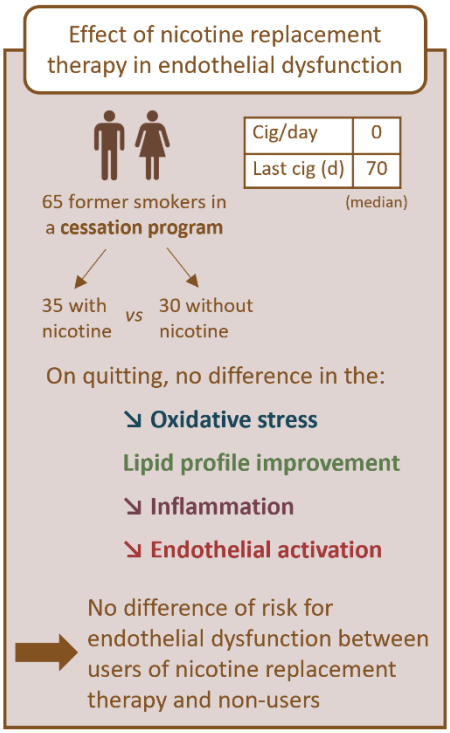
Abstract

Despite evidence on the health hazard of tobacco use, smoking remains a leading cause of preventable disease and death and a major cause of cardiovascular diseases (CVDs) worldwide. Although the mechanisms by which cigarette smoking induces and promotes CVDs are complex and not clearly elucidated, several key pathways are suggested: inflammation, oxidative stress, and altered lipid metabolism could be responsible for endothelial dysfunction, an early precursor of CVDs. In this thesis, we aimed to evaluate the effect of smoking, but also of nicotine replacement therapy (NRT) for cessation, on endothelial dysfunction. We addressed the involved processes by the quantification of specific biomarkers in smokers in comparison with non-smokers. We showed that endothelial activation, inflammation, and oxidative stress could be increased in smokers. Lipid profile was also altered in smokers. These observations could objectify the increased risk for smokers for CVDs. We followed the changes in biomarkers concentrations for smokers on quitting in order to determine if smoking-induced adverse effects on endothelial function could be reversible. We found that endothelial activation, inflammation, and oxidative stress seemed to be reduced for quitters. Lipid profile was also improved. These effects could motivate smokers to quit, even more because they were already visible at short term (less than seventy days). Finally, we assessed the influence of NRT on biomarkers concentrations on quitting. Since the role of nicotine in CVDs development is still unclear and largely controversial in the literature, prescribers may hesitate to provide NRT while they are effective smoking cessation tools. We compared the changes in biomarkers concentrations on quitting for NRT users or non-users. It allowed us to demonstrate that NRT did not affect negatively the endothelial dysfunction-related biomarkers, which could encourage their prescription.


Biomarkers
Blood
Serum
Plasma
Urine
Tobacco exposure
CO
Nicotine Cotinine 3HC
Anatabine Anabasine
Oxidative stress
Iron
Uric acid
GPX SOD
Vitamins A/E/C
MDA
CoQ10
β-carotene
Anti-oxLDL
New analytical methods development
Endothelial function
ADMA
sVCAM
sICAM
Inflammation
CRP
MCP-1
IL-6/8/10
TNF-α
Lipids
Cholesterol
HDL LDL
Triglycerides
Lp(a) sdLDL
ApoA1/B


Effect of smoking on endothelial function
83 non-smokers
vs
138 smokers
Cig/day 20
Last cig (h) 1.5
(median)
Tobacco biomarkers ↗
Oxidative stress ↗ (MDA ↗, uric acid, β-carotene, vit C ↘)
Lipid profile alteration (triglycerides ↗)
Inflammation ↗ (IL-8, CRP ↗)
Endothelial activation ↗ (sICAM ↗)
Endothelial dysfunction ↗
Risk for CVD ↗


Effect of smoking cessation in endothelial dysfunction
65 smokers in a cessation program
Baseline
vs
Post-quit
Cig/day 20
Last cig (h) 1.5
Cig/day 0
Last cig (d) 70
(median)
Tobacco biomarkers ↘
Oxidative stress ↘ (uric acid ↘)
Lipid profile improvement (HDL, ApoA1 ↗, LDL, sdLDL ↘)
Inflammation ↘ (IL-6, CRP ↘)
Endothelial activation ↘ (sICAM ↘)
Endothelial function improvement
Risk for CVD ↘


Effect of nicotine replacement therapy in endothelial dysfunction
65 former smokers in a cessation program
Cig/day 0
Last cig (d) 70
(median)
35 with nicotine
vs
30 without nicotine
On quitting, no difference in the:
↘ Oxidative stress
Lipid profile improvement
↘ Inflammation
↘ Endothelial activation
No difference of risk for endothelial dysfunction between users of nicotine replacement therapy and non-users

Abbreviations

ACT: aqueous extract of cigarette tar

ADMA: asymmetric dimethylarginine

cGMP: cyclic guanine monophosphate

COPD: chronic obstruction pulmonary disease

CRP: C-reactive protein

CVDs: cardiovascular diseases

CYP2A6: cytochrome P450 2A6

DNA: deoxyribonucleic acid

EDHF: endothelium-derived hyperpolarising factors

EDRF: endothelium-derived relaxing factor

EDV: endothelium-dependent vasodilation

ELISA: enzyme-linked immunosorbent assay

EMA: European Medicines Agency

ENDS: electronic nicotine delivery devices

eNOS: endothelial nitric oxide synthase

ESI: electrospray ionisation

ETS: environmental tobacco smoke

FMD: flow-mediated dilation

FMO3: flavin-containing monooxygenase 3

GABA: γ-aminobutyric acid

GPX: glutathione peroxidase

GSH: glutathione

3HC: trans-3'-hydroxycotinine

HDL: high-density lipoprotein

HNE: 4-hydroxy-nonenal

HPLC: high-performance liquid chromatography

ICAM: intercellular adhesion molecule

IL: interleukin

8-iso-PGF2α: 8-iso-prostaglandin F2α

LDL: low-density lipoprotein

LOD: limit of detection

LOQ: limit of quantification

MCP-1: monocyte chemoattractant protein 1

MDA: malondialdehyde

nAChRs: nicotinic cholinergic receptors

NADPH: nicotinamide adenine dinucleotide phosphate (reduced form)

NMR: nicotine metabolite ratio

NO: nitric oxide

NRT: nicotine replacement therapy

oxLDL: oxidised low-density lipoprotein

PAHs: polycyclic aromatic hydrocarbons

PUFAs: polyunsaturated fatty acids

RNS: reactive nitrogen species

ROS: reactive oxygen species

SMC: smooth muscle cells

SOD: superoxide dismutase

SR: sustained-release

TNF-α: tumor necrosis factor-α

TSNAs: tobacco-specific nitrosamines

UHPLC: ultra-high performance liquid chromatography

VCAM: vascular cell adhesion molecule

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INTRODUCTION

1. Tobacco smoking

Tobacco use is a practice that leads to psychological addiction, which is related to tobacco use behaviour, and physiological addiction, which is mainly due to nicotine – the active component in tobacco [1,2]. Several forms of tobacco consumption exist and can be divided into the use of combustible and noncombustible tobacco products. Combustible tobacco products are mostly cigarettes, but also cigars, cigarillos, water pipes, and pipes. Noncombustible tobacco products include electronic cigarettes and other tobacco formulations for chewing, dipping, or snuffing [1,3,4].

Cigarette remains the choice of tobacco use globally because of its accessibility [1]. When smoking cigarettes, blood concentration of nicotine rises quickly and peaks at the smoking completion. Nicotine is rapidly absorbed from cigarette smoke through the lungs and high levels of nicotine reach the brain after 10-20 seconds, which is faster than with intravenous administration. Cigarette smoking is the most reinforcing and dependence-producing form of tobacco use due to this rapid increase in nicotine levels [1,5]. It is now well known that epidemiologic impact and adverse health effects of cigarette smoking are important. Therefore, it is imperative to reduce the prevalence of cigarette smoking and the resultant smoking-induced diseases and deaths [6].

1.1 Composition of cigarette smoke

Tobacco smoke consists in an aerosol of solid/liquid droplets – called “particulate phase” – in a gaseous phase. It is generated by complex and overlapping processes: burning, pyrolysis, pyrosynthesis, distillation, sublimation, and condensation [7,8].

When smoking cigarette, a complex mixture is inhaled into the respiratory system. This smoke emerging from the mouth end of the cigarette during puffing is called “mainstream smoke”. “Sidestream smoke” has also to be considered when evaluating cigarette smoke toxicity. This one is emerging into the environment from the lit end of the cigarette between the puffs [7]. The mixture between exhaled mainstream smoke and sidestream smoke in the environment after dilution and aging forms the environmental tobacco smoke (ETS),

also called “secondhand tobacco smoke” or “passive smoke” (Figure 1) [9–11]. In general, the same constituents are present in mainstream smoke and sidestream smoke but their levels can differ, reflecting in this way that chemical influences are more complex than just changes in puff frequency [9,12]. Actually, cigarette smoke is a complex and dynamic system: particle size and thus relative amounts of chemicals can change depending on the concentration of smoke, the temperature, and the time after it leaves the cigarette. Levels of both mainstream and side-stream emissions can also vary according to the specific properties of the tobacco and the physical design of the cigarette [12,13].

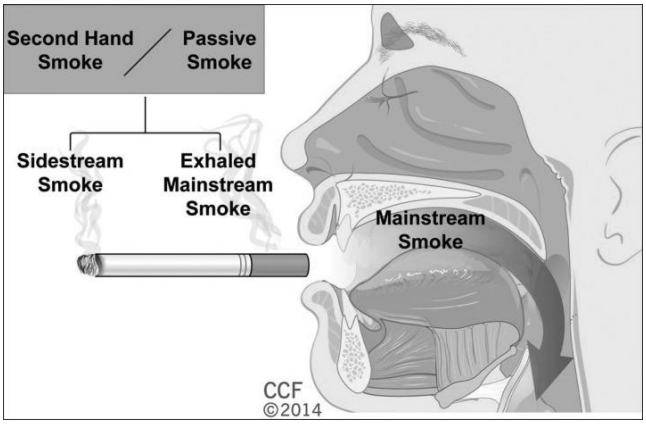


Figure 1: smoke types produced when smoking cigarette [10].

1.1.1 Chemical composition of mainstream smoke

When a cigarette burns, oxygen reacts with carbonised tobacco inside the combustion zone (Figure 2) to produce carbon dioxide, carbon monoxide and hydrogen. At the puffing, the temperature reaches up to 950°C. Downstream of the combustion zone, the main part of the 5000 chemicals in smoke are generated in the cooler pyrolysis/distillation/pyrosynthesis zone. It forms a super-saturated vapor which rapidly cools – within a few milliseconds – in the tobacco rod and condenses into the aerosol particles that make up the mainstream smoke [7,9].

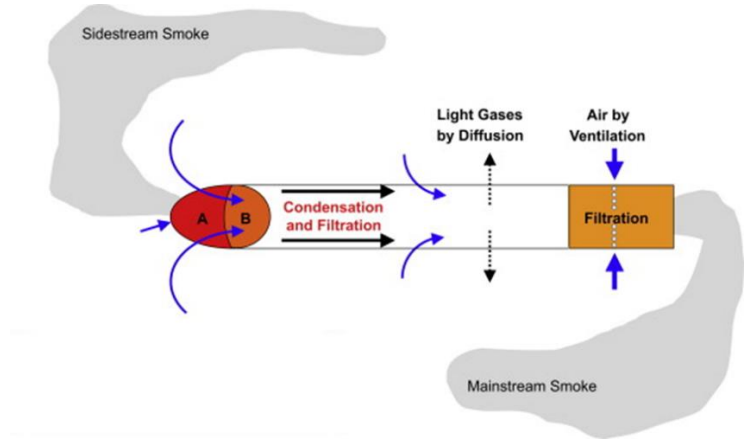


Figure 2: burning cigarette. A = combustion zone; B = zone of distillation, pyrolysis, and pyrosynthesis [9].

The generated smoke is composed of nitrogen, oxygen, the particulate phase, and the vapor phase (Figure 3) [9]. The smoke condensate free of water and nicotine is called “tar”. Several compounds, such as the polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines (TSNAs), phytosterols, and the metals are nearly only present in the particulate phase. Others, for example phenols and cresols are semi-volatiles and, therefore, are partitioned between the particulate and gaseous phases [9,14]. A number of combustion products, such as carbon monoxide, carbon dioxide and nitric oxide are found in the gaseous phase exclusively. Other components known for their toxic or carcinogenic properties are also in this phase, such as 1,3-butadiene, formaldehyde, acetaldehyde, acrolein, benzene and hydrogen cyanide [7,9]. Currently, approximately 5000 different compounds have been identified in fresh tobacco smoke and nearly all classes of organic chemicals can be found, including traces of metals [15].

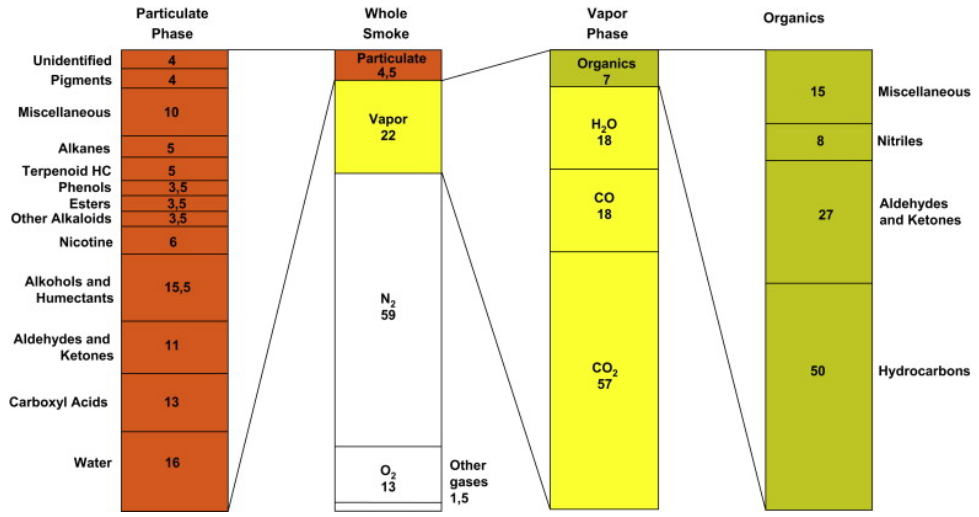


Figure 3: approximate chemical composition of whole cigarette smoke [9].

Mainstream smoke composition and smoke yield highly depend on the amount of tobacco in the cigarette, the cigarette physical dimensions, and the filter type used [12]. Besides modifying the particulate phase by retaining some particles, the ventilated filter dilute the smoke with air, affecting tar, nicotine, and carbon monoxide yields [7,16].

There are around 10^9-10^{10} particles per cubic centimeter in fresh mainstream smoke. The particles size is from 0.1 to <1 µm in diameter and they can reach the deep lung [9,17].

1.1.2 Chemical composition of sidestream smoke

For the majority of cigarette worldwide, mainstream smoke is slightly acidic, while sidestream smoke is slightly alkaline, due to a high amount of ammonia [18]. Unaged particles in sidestream smoke are reported smaller than those in mainstream smoke with an average diameter of 0.1-0.2 µm [19]. It should be noticed that a mass loss of 25% of these particles can be observed when they become part of ETS [20].

Besides differences of composition and concentration between sidestream smoke, mainstream smoke and ETS, differences in their acute toxicity and genotoxicity have been shown [7].

1.2 Health effects of tobacco smoking

With a contribution of around 18% of all deaths, tobacco use is one of the leading causes of premature mortality in the world. Four main groups of diseases related to tobacco use are reported the main causes of death and disability in Europe: cardiovascular diseases, cancers, respiratory diseases and diabetes [21–24].

Tobacco causes adverse health effects not only in smokers, but also in individuals exposed to secondhand smoke. The effects depend on the duration of smoking over years and the exposure to cigarette smoke [1]. The mechanisms by which they occur involve multiple complex steps being part of oxidative stress, inflammation, DNA damage and endothelial dysfunction [25].

1.2.1 Cardiovascular diseases

A relationship between cigarette smoking and cardiovascular events has been clearly identified [1,26]. Mechanisms involved are not yet well understood but they include endothelial dysfunction, oxidative stress, inflammation, prothrombic effects, altered lipid metabolism, increased demand for myocardial oxygen and blood, decreased supply of myocardial blood and oxygen, and insulin resistance [11]. Cigarette smoking and secondhand smoke exposure are reported major causes of coronary heart disease, stroke, aortic aneurysm, and peripheral arterial disease [26]. This topic will be addressed in detail further (see point 6).

1.2.2 Cancers

Smoking is the most preventable cause of cancer-related deaths [1,27]. Cancer results from the presence of carcinogens in cigarette smoke, including many polycyclic aromatic hydrocarbons (PAHs). PAHs are composed of two or more fused benzenoid rings and are known for their carcinogenic and mutagenic properties. The most potent carcinogen among PAHs is benzo[a]pyrene [28,29]. Carcinogens bind to DNA and lead to DNA damage and gene mutations. It stimulates uncontrolled cell growth and inhibits normal mechanisms restraining cell growth and spread [30]. Up to now, causal relationship between cigarette

smoking and several cancers has been established: lung, head, neck, liver, bladder, cervix, oesophagus, colon, and rectum cancers [1,27]. There is also evidence for a moderate increase in the risk of breast cancer in women who smoke tobacco [31]. However, more research is needed to conclude that ETS exposure increases risk of breast cancer in non-smokers [31,32]. Cigarette smoking may increase the risk of prostate cancer and the risk of dying from this cancer is increased in smokers [1,27,33].

1.2.3 Respiratory diseases

Cigarette smoking is involved in the development of chronic pulmonary diseases, such as chronic obstruction pulmonary disease (COPD). COPD is a major cause of morbidity and mortality and it has been recognised that smoking is its most important causative factor [34,35]. Responsible mechanisms are loss of cilia in the lungs, mucus hyperplasia, and overall inflammation. It leads to abnormal functioning of the lung and injury. Smoking is also associated with an exacerbation of asthma in adults due to chronic airway inflammation, impaired mucociliary clearance, increased bronchial hyperresponsiveness, greater allergic sensitisation, and increased production of IgE. Moreover, smoking increases the risk of contracting and dying from tuberculosis [1,34].

1.2.4 Diabetes

The risk of developing type 2 diabetes is 30-40% higher in smokers than in non-smokers. In addition, smokers with diabetes present higher risk of developing complications, such as nephropathy, blindness, peripheral neuropathy, and amputations [1,36,37].

1.2.5 Other effects

Smoking is also associated with many other dysfunctions, which not allow to be comprehensive. It has been shown that smoking causes several reproductive abnormalities in both men and women. Erectile dysfunction in men has been clearly associated with smoking while some studies discuss the possible effects of smoking on male fertility [1,10]. In women, a decreased fertility has been observed. Maternal tobacco smoking can result in troubles concerning the pregnancy (for example ectopic pregnancies, miscarriages, and

premature births) or the baby (including low birth weight, orofacial clefts, and sudden infant death syndrome) [1,38,39].

Smoking impairs immune function, which can increase the risk of pulmonary infections and rheumatoid arthritis. The risk of peptic ulcer disease can also be increased due to the impact of smoke on the gastrointestinal tract. In postmenopausal female smokers, there is an increased risk of hip fractures and low bone mineral density. As previously broached, secondhand smoking is also responsible for negative health effects, including low-birth rate in offspring of exposed mothers, sudden infant death syndrome, and type 2 diabetes [1,36].

2. Nicotine

Nicotine is the principal alkaloid found in the tobacco plant and the major responsible for tobacco addiction [40]. An average tobacco rod contains 10-14 mg of nicotine and provides about 1-1.5 mg of absorbed nicotine when smoked. It is mainly the (S)-isomer of nicotine (Figure 4) which is found in tobacco; only 0.1-0.6% of total nicotine content is (R)-nicotine [5,41].

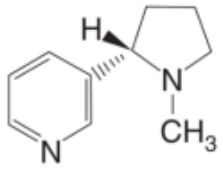


Figure 4: structure of (S)-Nicotine [5].

2.1 Nicotine pharmacology and tobacco addiction

When smoking tobacco, nicotine is carried in smoke particles into the lungs where it is absorbed rapidly into the pulmonary venous circulation. Then, it passes into the arterial circulation and reaches the brain quickly [42]. Nicotine diffuses readily into brain tissue where it binds to nicotinic cholinergic receptors (nAChRs). These receptors are ligand-gated ion channels and allow the entry of cations – sodium and calcium [41]. When nicotine stimulates these receptors, it results in the release of several neurotransmitters in the brain, mainly dopamine, which is critical in drug-induced reward [43]. Other neurotransmitters are also released, such as norepinephrine, acetylcholine, serotonin, γ-aminobutyric acid (GABA), glutamate, and endorphins, and mediate various behaviors of nicotine [41].

Repeated exposure to nicotine leads to tolerance to some effects of nicotine but also to an increase in the number of nAChR binding sites in the brain. This increase could arise from upregulation in response to nicotine-mediated desensitisation of receptors [41]. The desensitisation may play a role in nicotine tolerance and dependence [44]. Usual smokers maintain near-complete saturation and thus desensitisation of brain nAChRs to avoid nicotine withdrawal symptoms. Moreover, in a complementary way, smokers can also

continue to obtain some rewarding effects from the conditioning associated with smoking, such as the taste and feel of the smoke. These two components have to be considered in the addiction phenomenon [41,44,45].

Nicotine withdrawal brings about a negative emotional state, such as anxiety, difficulty concentrating, irritability, and increased stress, which may greatly contribute to relapse to tobacco use [1,41].

2.2 Metabolism

2.2.1 Nicotine absorption

Nicotine is a weak base – pKa = 8 – and its absorption across biological membranes depends on pH. It does not rapidly cross membranes when present in its ionised state, such as in acidic environments [42]. The pH of tobacco smoke and thus the proportion of nicotine absorbed depend on the smoke composition and the tobacco curing method used. The pH of smoke of most cigarettes sold in Europe is slightly acidic (pH 5.5-6.0), resulting in low nicotine absorption [5,41,46]. In the early 1970s, tobacco companies started using ammonia compounds to increase smoke pH values and facilitate rapid pulmonary absorption [47]. However, researches do not indicate that ammonia-laced tobacco results in significant higher blood levels of nicotine. This is probably due to the fluid in the lungs acting as a chemical buffer. Thereby, main sites of nicotine absorption in the lungs seem unaffected by ammonia in inhaled smoke [47,48].

Nevertheless, smoking is a highly efficient form of nicotine administration given that it enters the circulation rapidly through the lungs and moves into the brain within a few seconds [42]. Moreover, inhalation enables nicotine to skip the first-pass intestinal and hepatic metabolism. This rapid rate of absorption and entry into the brain makes the nicotine rush greater and thus gives it reinforcing properties. Smoking process also allows the smoker to titrate precise dose to obtain desired effects [41].

2.2.2 Nicotine metabolism

Nicotine is extensively metabolised by the liver to produce a number of metabolites. There are six primary metabolites identified (Figure 5) [42]. The most important metabolite of nicotine is cotinine: about 70-80% of nicotine is converted to cotinine [49]. This transformation is performed in two steps: (i) the formation mediated by CYP2A6 of the intermediate nicotine-$\Delta^{1'(5')}$-iminium ion, which is in equilibrium with 5'-hydroxynicotine; (ii) the formation of cotinine catalysed by a cytoplasmic aldehyde oxidase [42]. Another primary metabolite of nicotine is nicotine N'-oxide which requires the action of a flavin-containing monooxygenase 3 (FMO3). Only 4-7% of nicotine absorbed is metabolised via this route [50]. Nicotine can also be metabolised by two non-oxidative pathways: methylation of the pyridine nitrogen giving nicotine isomethonium ion and glucuronidation. Nicotine glucuronidation represents 3-5% of nicotine conversion and is catalysed by uridine diphosphate-glucuronosyltransferase (UGT) enzyme(s). A minor pathway in the metabolism of nicotine is oxidative N-demethylation to generate nornicotine. It can be noted that nornicotine is a constituent of tobacco leaves but the majority of urine nornicotine arises from the metabolism of nicotine rather than directly from tobacco. Finally, a new metabolic pathway was recently reported leading to the formation of 2'-hydroxynicotine [5,41,42].

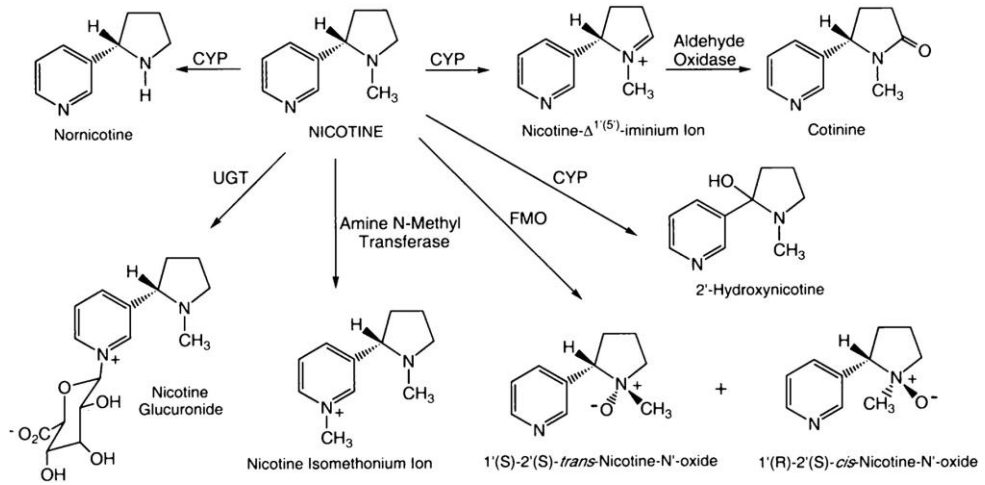


Figure 5: primary routes of nicotine metabolism [42].

2.2.3 Cotinine metabolism

Although the conversion to cotinine is the main metabolic pathway of nicotine, only 10-15% of the nicotine absorbed is found as unchanged cotinine in smokers' urine [51]. Actually, cotinine is also metabolised to six reported primary metabolites (Figure 6) [42]. The most of the identified urinary metabolites of nicotine are derived from cotinine. 3'-hydroxycotinine is the main nicotine metabolite detected in smokers' urine. The conversion of cotinine to 3'-hydroxycotinine is catalysed by CYP2A6 enzyme and is highly stereoselective for the trans-isomer. 3'-hydroxycotinine is also excreted as a glucuronide conjugate. About 40-60% of the nicotine dose is found as 3'-hydroxycotinine and its glucuronide conjugate in urine. Cotinine N-oxide is formed by P450 enzymes and accounts for 2-5% of the nicotine in urine. Cotinine methonium ion can also be excreted in urine. About 1% of total nicotine is detected as norcotinine in urine. This metabolite is formed by two pathways: demethylation of cotinine catalysed by CYP2A6 enzyme or oxidative metabolism of nornicotine. Some studies suggest that demethylation of cotinine giving norcotinine presents an intermediate step with the formation of N'-hydroxymethylnorcotinine. Cotinine can also be converted to 5'-hydroxycotinine with CYP2A6 enzyme as catalyst. The concentration of 5'-hydroxycotinine in urine corresponds to a ratio of 3.7% to the excreted 3'-hydroxycotinine. The last metabolite of cotinine – and thus of nicotine – is the glucuronide conjugate of cotinine [42,52,53].

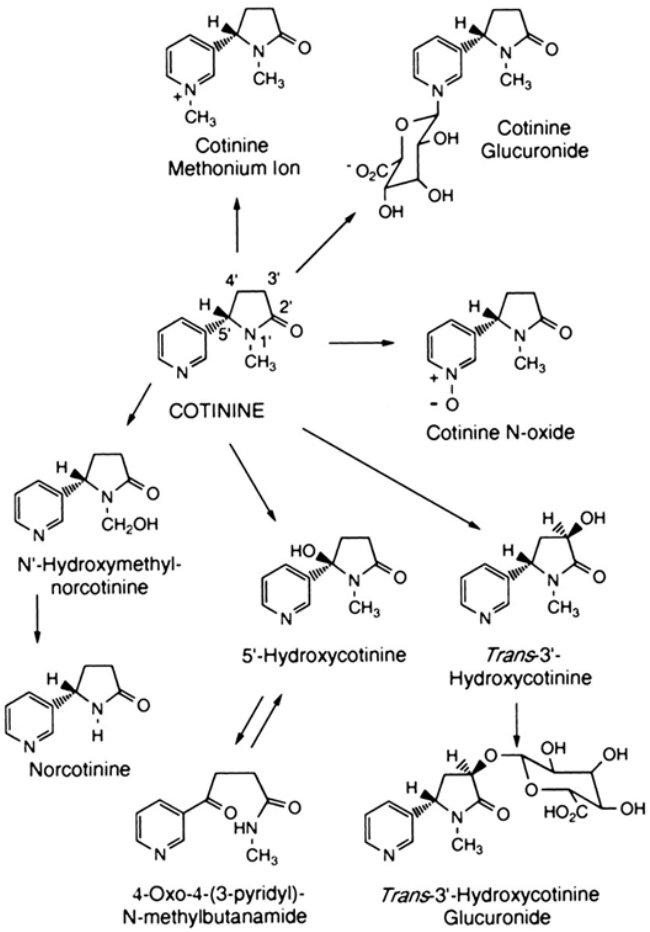


Figure 6: pathways of cotinine metabolism [42].

2.2.4 Rates of nicotine and cotinine metabolism

The total clearance of nicotine is about 1200 ml/min while metabolism of cotinine is slower with a clearance about 45 ml/min. The clearance of trans-3'-hydroxycotinine is also quite slow and is about 82 ml/min [5].

3. Treatments for smoking cessation

For different reasons, including for decreasing the risk of developing tobacco-related diseases, some smokers begin a cessation program. However, cigarette smoking remains a difficult habit to break. Many smokers must try several times before they can successfully quit and many smokers are unable to stop on their own. It is important to note that a behavioural support is crucial for smoking cessation. A wide variety of smoking cessation methods exist to help the smoker in this goal [1,54]. Counseling and medication are effective for treating tobacco dependence, but is was shown that the combination of these two tools is more effective than either alone. Therefore, clinicians are encouraged to recommend all smokers making a quit attempt to use both counseling and medication [55].

Depending on the guidelines [55–58], there are six (or seven) first-line medications reported for smoking cessation, whose five are nicotine and one (or two) non-nicotine: transdermal nicotine patch, nicotine gum, nicotine tablet, nicotine inhaler, nicotine nasal or oral spray, varenicline, (and bupropion sustained-release (SR)). It was shown that they reliably increase long-term smoking abstinence rates. If no effect is observed or in case of contraindication, second line agents – including clonidine and nortriptyline, and depending on the guidelines, buproprion SR – may be prescribed to the patient. They were also shown effective in tobacco cessation. Cytisine is another aid for smoking cessation. Finally, combination therapy of pharmacologic agents can be suggested when monotherapy turns out ineffective. It consists in adding short acting nicotine replacement therapy – nicotine gum, lozenge, inhaler, or nasal/oral spray – to longer acting agents, such as nicotine patch or bupropion SR [1,55]. Thereby, many treatments for smoking cessation exist and their recommendation varies according to the clinician. In Belgium, treatments based on nicotine or varenicline are the most commonly used [59].

3.1 Nicotine replacement therapy

Nicotine replacement therapy (NRT) products allow to reduce severity and duration of withdrawal symptoms by partially replacing nicotine obtained by tobacco use [60]. Several forms of NRT exist: nicotine gum, nicotine tablet, nicotine nasal/oral spray, nicotine inhaler,

and the transdermal patch. These multiple formulations of NRT allow the smoker to choose the route of administration, which may positively influence his adherence to treatment. The transdermal patch releases the nicotine dose continuously over a 16 or 24 hours period, while the other formulations are short-acting NRT giving the opportunity of dose self-titrating [61]. Various forms are available without a medical prescription and can be purchased in pharmacies. Studies have shown that NRT increases rate of quitting smoking by 50-70% and that all forms of NRT produce similar quit rates [62]. They are broadly well tolerated with mild adverse effects. Adverse effects depend generally on formulation and the delivery system used [1,61–63]. Common effects reported are irritation at the site of administration (throat or skin) and hiccups or nausea in case of oral administration [64].

Formulations of NRT are usually buffered to alkaline pH to facilitate nicotine absorption through mucous membranes. This absorption is slower and the increase of nicotine blood levels is more gradual than from smoking. This slow increase in blood and especially in brain levels allows to limit the risk of NRT abusing [5]. However, for this reason, NRT does not completely eliminate all symptoms of withdrawal. A more rapid delivery of nicotine is possible with nasal or oral spray [61]. It is also important to note that the use of nicotine gum, tablet, nasal/oral spray, and inhaler leads to the swallow of a portion of the nicotine dose which is subjected to first-pass metabolism. The bioavailability for these products is, therefore, about 50-80% [5,61].

3.2 Bupropion sustained-release (SR)

Bupropion is an atypical antidepressant that has been shown effective for tobacco dependence treatment [63]. Despite its antidepressant properties, this agent was not shown more effective among smokers who have depression compared with non-depressed smokers. Its mechanism of action in smoking cessation is not well understood, but it is known that bupropion is an inhibitor of dopamine and norepinephrine reuptake. It also acts as a weak agonist of nicotinic receptors [1,61]. Studies has shown that bupropion doubles the chances of quitting smoking compared with placebo and that withdrawal symptoms and cravings are decreased. It was also shown that it significantly increases long-term

(≥6 months) smoking abstinence. Quit rates appear similar when using NRT or bupropion. The most common adverse effects when using bupropion are insomnia – occurs in 30-40% of patients – and dry mouth – occurs in 10% of patients. Nausea can appear, with a similar incidence to the one caused by NRT. A risk of seizures and allergic reactions have also been observed with this treatment [61].

3.3 Varenicline

Varenicline is a partial agonist specific for the neuronal nicotinic acetylcholine receptor subtype $\alpha_4\beta_2$ ($\alpha_4\beta_2$-nAChR), which plays a central role in nicotine addiction. It stimulates receptor-mediated activity, but at a lower level than nicotine [61]. Moreover, it stimulates dopamine turnover and therefore relieves nicotine cravings and withdrawal symptoms [1]. It is also useful for patients who relapse after their quit day: the competitive binding of varenicline to $\alpha_4\beta_2$-nAChR inhibits dopaminergic activation when smoking, thus preventing a pharmacologic reward usually experienced [61]. It has been reported that the odds of quitting with varenicline were about 2.5 times that of placebo, and approximately 1.7 times better than with bupropion. The highest odds ratio for long-term smoking abstinence is found with varenicline compared with other available monotherapies. Generally, varenicline is well tolerated [61,63]. Most common adverse effects are nausea and abnormal sleep related events (abnormal dreams, nightmares, or somnambulism) [65]. The risk of varenicline abuse is low because a ceiling effect is observed when using partial agonists, i.e. increasing the dose beyond a certain point does not increase the effect [61].

Unfortunately, there is currently a shortage of Champix® (Pfizer), the medicine that contains the active substance varenicline. This shortage has been reported by the European Medicines Agency (EMA) and affects all Europe member states where Champix® has been marketed. Therefore, alternative treatments have to be proposed to patients [66].

3.4 Cytisine

Cytisine, a plant alkaloid, is a selective partial agonist at $\alpha_4\beta_2$-nAChR, such as varenicline. It has been licensed and used as a smoking cessation aid in some eastern and central

European and central Asian countries for more than 50 years. It was found to be more effective than placebo and NRT in aiding abstinence for at least 6 months. Cytisine seems to be well tolerated with no serious safety signals. However, it is associated with more adverse effects than NRT treatment. They include mild or moderate nausea, vomiting, dyspepsia, upper abdominal pain and dry mouth [67,68].

3.5 Clonidine

Clonidine is an α_2-adrenergic agonist used for the treatment of hypertension but it has shown some efficacity in smoking cessation. It seems to counteract central nervous system features of nicotine withdrawal, including craving and anxiety. It was demonstrated that clonidine approximately doubled the rate of abstinence after ≥12 weeks of follow-up compared with placebo. However, this treatment is limited by its adverse effects which are dose-dependent. They include significant sedation, postural hypotension, dry mouth, and constipation. Moreover, patients who stop clonidine treatment abruptly may undergo agitation, headache, tremor, and rebound hypertension [1,61].

3.6 Nortriptyline

Nortriptyline is a tricyclic antidepressant whose the action in smoking cessation is not well understood [1]. Several plausible mechanisms have been suggested: (i) the antidepressant use could prevent the high relapse rates due to the mutual symptom exacerbation of smoking cessation and depression, (ii) effects of nicotine could be replaced by the nortriptyline's noradrenergic effects, and (iii) nortriptyline could be a nicotine receptor antagonist. However, there are no studies supporting any of these potential mechanisms [69]. Nortriptyline seems to be as effective as NRT or bupropion given that it doubles the odds of smoking cessation compared with placebo. Nevertheless, it has been evaluated in a much smaller number of smokers than the other two. The most common adverse events related for nortriptyline include anticholinergic effects, such as dry mouth, constipation, and sedation. But these effects seem less common at the doses used for smoking cessation than at the ones used for treating depression. Potential cardiovascular effects can also be observed, such as arrythmia, hypertension, orthostatic hypotension, and tachycardia. In

case of treatment abruptly stopped, withdrawal symptoms such as nausea, headache, and malaise may occur [61]. The nortriptyline's safety profile has to date prevented its classification as a first-line agent for smoking cessation because of the absence of more extensive testing [69].

4. Tobacco biomarkers

Assessing the tobacco smoke exposure associated with health risks constitutes an active area of research. Accurate measurement of tobacco exposure is crucial to estimate reliably these risks [70]. However, the exposure self-report leads to various biases [71]. For example, some groups of people are reluctant to disclose their smoking status and exposure to tobacco – such as pregnant women and parents of young children – or for others, recall of tobacco exposure may also prove difficult. For this reason, the use of biomarkers of tobacco smoke exposure was suggested. Currently, they constitute the most commonly used objective method of assessing tobacco smoke exposure. It consists in measuring concentrations of components of smoke in the body or in the exhaled air of exposed individuals [70,71].

4.1 Features of biomarkers

A biomarker is an exogenous substance or its metabolite that can be measured in the body in order to predict the incidence of an outcome – exposure or disease. An ideal biomarker of tobacco smoke exposure should be specific to tobacco combustion and have a long half-life in the body. It should be (i) able to relate a prior exposure, (ii) the agent associated with health effects – or be strongly associated with such an agent, (iii) detectable in trace amounts as well as in high levels with high precision, (iv) dose dependent, (v) measurable by noninvasive methods in readily collected biospecimens – i.e. saliva, urine, and blood – in which it is stable upon storage, and (vi) inexpensive to assay [70,72]. Several tobacco smoke exposure biomarkers have been proposed and include carbon monoxide, carboxyhaemoglobin, nicotine and its metabolites, thiocyanate, DNA adducts, protein adducts, even other tobacco alkaloids [70,73]. However, carbon monoxide, carboxyhaemoglobin, thiocyanate, DNA adducts, and protein adducts are not sufficiently specific for tobacco smoke exposure and/or have not long half-life resulting in an increase of false negatives [70].

4.2 Carbon monoxide used as a biomarker of combustion

Carbon monoxide (CO) is a byproduct of organic matter combustion. It is used as a biomarker of recent smoke absorption from combustible tobacco products (cigarettes, cigars, pipes, and hookah) but not smokeless tobacco or most electronic nicotine delivery devices (ENDS). The measurement of exhaled CO is a widely used method for assessing tobacco smoke exposure because it is easily performed with a relatively inexpensive portable device [71,74]. However, CO is not specific for tobacco smoke exposure since it is present in marijuana smoke, in ambient air containing common pollutants (for example automobile exhaust and smoke from barbecue), even it can also be produced in the human body. However, this endogenous formation only contributes for 1-2 ppm [71,75]. Suggested cutoff value for CO largely depends on the context of potential environmental exposures: a range of 0-5 ppm is typically admitted representing non-smokers in countries that ban smoking in public places and have strong regulations on air pollution while a range of 2-8 ppm is more suitable for countries without these regulations [71,76]. The half-life of CO is around 4 hours but is affected by pulmonary ventilation. Thereby, the half-life can be of 2 hours during exercise and 8-10 hours during sleep [71].

4.3 Alkaloids and metabolites used as biomarkers of tobacco smoke exposure

4.3.1 Nicotine

Nicotine is the most abundant alkaloid in tobacco – accounts for 95% of the alkaloid content – and was one of the first biomarkers used to assess exposure to cigarette smoke. The measurement reflects nicotine uptake, from tobacco or other non-tobacco nicotine delivery products, such as ENDS or NRT. However, its short half-life – approximately 2-3 hours in the blood – does not allow to reflect long-term exposure but only recent exposure. Moreover, considerable variability between individuals exists due to differences in nicotine uptake, metabolism, and elimination. When smoking, only 5-10% of inhaled nicotine is excreted unchanged in urine given that the rest is metabolised by the liver. Therefore, quantification method of nicotine must be very sensitive due to its small amount present in

body fluids. Nicotine can be measured in several matrices, such as plasma, saliva, and urine, depending on the characteristics of the study [70–73].

4.3.2 Cotinine

Cotinine – the main metabolite of nicotine – is the biomarker of choice for nicotine uptake. It is especially used for evaluating both tobacco smoking exposure and ETS exposure. The nicotine absorbed dose is best approximated by cotinine in the blood. However, blood cotinine can also be accurately estimated by measuring cotinine in urine or saliva given that cotinine concentrations in all biological fluids are highly correlated. Cotinine is found at detectable levels with sensitive analytical methods in several matrices, commonly in blood – serum/plasma – urine, and saliva [70,72,77].

Using cotinine as biomarker is better than nicotine because of its longer half-life varying from 8 to 30 hours due to genetic, hormonal, and other factors. Concentrations of cotinine throughout the day are more constant and do not oscillate as nicotine concentrations. Because of this stability in the blood over time and its ability to reflect long-term exposure, cotinine has become the preferred biomarker of nicotine exposure [70,71,77].

It should be noticed that some variations in the relation between cotinine concentrations in body fluids and nicotine intake exist. They are due to individual differences in nicotine metabolism and in cotinine clearance itself. However, this source of imprecision is not large and cotinine levels can be considered as accurate reflect of the nicotine exposure [70,77]. Some studies report urinary cotinine adjusted for creatinine in order to take into account, in part, differences in dilution effects given that urinary cotinine depends on renal function, flow rate, and urinary pH. However, creatinine excretion is also variable and for example, low creatinine in children relative to adults leads in cotinine to creatinine ratios indicating active smoking [70]. Therefore, many researchers continue to use unajusted cotinine values, especially since most urine cutoff values have been reported as unajusted [71].

When excluding sources of nicotine other than tobacco (for example NRT or ENDS), cotinine is a biomarker allowing to distinguish accurately between smokers and non-smokers and its

concentration allows to evaluate the nicotine impregnation among non-smokers exposed to ETS. Several reference intervals of cotinine in the most common matrices have been proposed [70,71,78], but they are variable depending on the authors and adequate validation and investigation of these cutoffs have not been undertaken. An example of cutoffs is shown in Table 1. Due to the variation of individuals' cotinine half-life, it is recommended measuring cotinine after 6-7 days to assess compliance with non-smoking and minimise false positivity [70,71].

Table 1: reference intervals of cotinine in plasma, urine, and saliva [70].

Matrix	**Unexposed nonsmokers**	**Passive smokers**	**Active smokers**
Plasma (ng/ml)	0.09-0.7	2-10	>10
Urine (ng/ml)	<10	10-100	>200
Saliva (ng/ml)	0-5	5-10	>10

4.3.3 Nicotine metabolite ratio (NMR)

Cotinine is metabolised by CYP2A6 to trans-3'-hydroxycotinine (3HC) (see point 2.2.3). The 3HC to cotinine ratio – called "the nicotine metabolite ratio" (NMR) – is used as an *in vivo* biomarker of CYP2A6 activity and correlates highly with nicotine clearance [79,80].

Nicotine metabolism rate is subjected to individual variations because it depends on CYP2A6, which is a genetically polymorphic enzyme. About 60% of the variability in nicotine clearance and about 40% of the variability in cotinine clearance is heritable. Reduced activity of CYP2A6 and null mutations are associated with slower nicotine clearance and are more prevalent among non-smokers compared with smokers. In smokers, reduced activity or null variants of CYP2A6 result in a lower dependence to nicotine compared with wild-type genotypes. Studies have demonstrated that smokers with faster CYP2A6 activity and,

therefore, higher NMR have higher tobacco consumption and lower odds of smoking cessation without any active pharmacological intervention [81,82]. CYP2A6 activity appears interesting to predict therapeutic response to treatments for smoking cessation – and thus to personalise treatment – but its skills as biomarker are diminished by: (i) the complexity and the costs of genotyping, (ii) the fact that all CYP2A6 variants are not yet identified, and (iii) the fact that CYP2A6 variants do not account for environmental influences on nicotine metabolism rate, such as oral contraceptive use. For these reasons, the use of NMR as biomarker of nicotine clearance has emerged. NMR takes into account CYP2A6 genetic variation, other genetic factors, and environmental factors known to influence nicotine clearance, such as age, race, hormonal factors, and smoking itself. Another advantage of this biomarker is that it can be assessed noninvasively, i.e. from saliva, plasma, or urine. It is highly reproducible and independent of time since last cigarette. Indeed, half-life of 3HC quantified alone is about 5 hours but at steady state, the levels of 3HC derived from cotinine are formation-dependent during smoking; thus, the half-life of 3HC is the same as that of cotinine and the NMR remains constant over time [79,81,83].

Several studies has examined the relationship between the NMR and the response to treatments for smoking cessation. Some of them showed that slow metabolisers of nicotine had significantly higher quit rates compared with fast metabolisers [84–87]. However, simply increasing the nicotine dose among fast metabolisers resorting to NRT did not increase quit rates significantly [88]. Others reported that bupropion enhanced quit rates for fast nicotine metabolisers while no added benefit was observed in slow metabolisers [89]. Similarly, a study showed that varenicline was more effective than NRT for faster nicotine metabolisers [90]. Finally, a study demonstrated a positive correlation between the use of nicotine tablets and the NMR [91]. All these results suggest that NRT is more effective for slower metabolisers, whereas non-nicotine medications are more effective for faster metabolisers. It could be explained by the fact that replacement nicotine may not produce the same dopaminergic effect for faster metabolisers because it is cleared more quickly while non-nicotine medication may better address dopaminergic activity, which is crucial to

quit successfully. With this in mind, the NMR could be used as a biomarker to personalise smoking cessation treatment [5,81,83].

4.3.4 Anabasine and anatabine

Cotinine is a biomarker for nicotine uptake, but it does not allow to distinguish between active smokers and NRT users. Expired carbon monoxide, carboxyhaemoglobin, and thiocyanate may be used to detect active smoking given that they are products of combustion. Nevertheless, as previously mentioned, these substances are less specific and less sensitive for detecting smoking. The solution to validate tobacco abstinence in NRT users is, therefore, using substances that are present in tobacco, measurable in biological fluids, but not derived metabolically from nicotine. Minor alkaloids, such as anabasine and anatabine, can play this role. They are present in tobacco and absent in nicotine-containing medications. Moreover, they are not likely present in foods or in other sources of exposure. Anabasine and anatabine in tobacco exist as mixtures of enantiomers (Figure 7). R-isomers represent 4-45% of the amount of these alkaloids in tobacco. The half-lives of anabasine and anatabine, based on urinary excretion data, were found to be 16 and 10 hours, respectively [92,93].

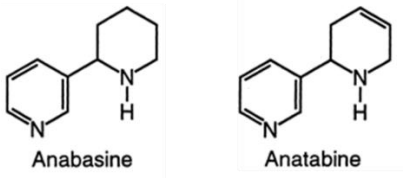


Figure 7: structures of anabasine and anatabine. They exist as mixtures of enantiomers in tobacco [92].

Two studies used the criterion of a concentration of >2 ng/ml in urine for both anabasine and anatabine to distinguish between active smokers and individuals undergoing NRT. Authors also reported mean concentrations found in urine of cigarette smokers: 22-24 ng/ml for anabasine and 22 ng/ml for anatabine. They noticed that anatabine concentrations in smokers' urine were about half those found in smokeless tobacco users' urine (41-45 ng/ml) despite similar nicotine and cotinine levels and the considerably lower

level of anatabine in smokeless tobacco products than in cigarette. A likely explanation is that anatabine could be decomposed to a much greater extent than is nicotine in burning tobacco, leading to a lesser absorption by cigarette smokers than by smokeless tobacco users. Authors suggest the validation of anabasine and anatabine as biomarkers during NRT because of their absence in NRT products [71,92].

4.4 Methods for quantifying nicotine, its metabolites and the minor tobacco alkaloids

Several analytical methods have been developed to quantify tobacco biomarkers [70,71]. Nicotine and its metabolites can be measured in the different matrices by four broad techniques: colorimetry, chromatography, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA). Colorimetry is the less specific method. Advantages and limitations of the other common techniques are summarized in Table 2.

Table 2: analytical methods for quantifying nicotine and its metabolites in biological samples [70].

Assay	**Advantages**	**Disadvantages**
Chromatography	- High sensitivity and specificity - Simultaneous assay of several biomarkers - Quantification limit: <0.1 ng/ml - Adequate for ETS detection	- Expensive - Extensive sample preparation
Radioimmunoassay	- High sensitivity and specificity - Easy to perform - Low sample volume required - Adequate for ETS detection	- Time consuming - Disposal of scintillation fluid (radioactive) - Depends on antisera - Costly equipment - Cross-reactivity with trans-3'-hydroxycotinine
Enzyme-linked immunosorbent assay (ELISA)	- Short analysis time - No radioactive compounds - Adequate for ETS detection	- Cross-reactivity with trans-3'-hydroxycotinine - Interference due to nonspecific antigen-antibody reaction

Among these techniques, the ultimate standard of reference in analysis nicotine and its metabolites in various body fluids is gas or liquid chromatography-mass spectrometry because of its high sensitivity [70].

High performance liquid chromatography (HPLC) is a technique allowing the separation, identification and quantification of components in a mixture, as components in a biological sample. A HPLC system is composed of a stationary phase (solid), a mobile phase (liquid) of varying polarity, and a detector (Figure 8). The compounds injected in the mobile phase move through the stationary phase and interact to varying degrees with this stationary phase, resulting in separation and different "retention times" [94]. There is no universal detector, therefore, it is selected according to the nature of the compounds of interest. The most used detectors are based on the measurement of absorbance in the ultraviolet and visible spectrum, the revealing fluorescence or electrochemical techniques [95]. The coupling of mass spectrometry to HPLC for compounds identification and quantification is also possible. It is widely recognised as a powerful analytical tool and it is based on the measurement of the mass-to-charge ratio of ions [96]. Electrospray ionisation (ESI) is the most commonly used ions source in methods for nicotine and its metabolites quantification [97]. The liquid containing the compounds of interest is dispersed by electrospray into a fine aerosol of charged droplets. It results in gas phase ions after solvent evaporation and droplet fission [98].

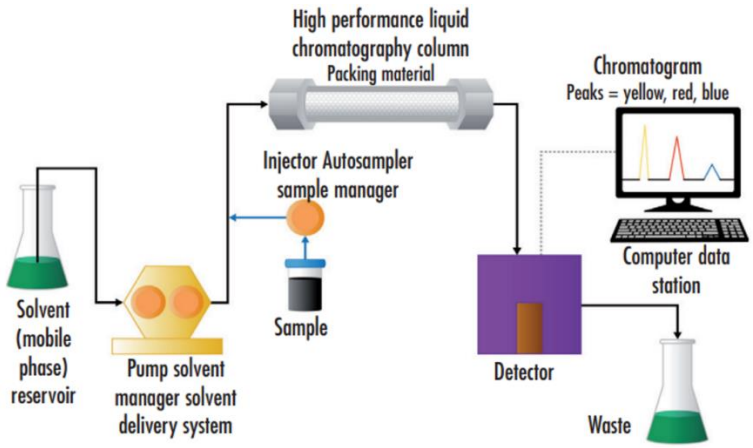


Figure 8: high performance liquid chromatography (HPLC) system. A high pressure pump is used to generate a specified flow rate of mobile phase. The sample is injected into the mobile phase and is carried into the chromatography column containing the stationary phase. The separation of the sample components is achieved through their various degrees of interaction with the column. Each component eluting from the column is then detected and a chromatogram is obtained [94].

Among body fluids, saliva appeared as the matrix of choice for assessing the concentration of nicotine and its metabolites in humans exposed to cigarette smoke. Sample collection is simple, convenient, and stress-free. It was considered less intrusive than urine collection and less painful or traumatic compared to blood collection [99,100]. However, a study showed that salivary cotinine levels tend to fluctuate considerably and are susceptible to many factors, especially when quantified by ELISA [100]. Moreover, collecting sufficient volumes of saliva seems more complicated than collecting urine. Urinary cotinine concentration appears as a more accurate biomarker for evaluating tobacco smoke exposure [99,100].

Due to their low concentrations in body fluids, the minor tobacco alkaloids – anabasine and anatabine – require a sensitive technique of quantitation [71]. Several studies have developed methods of liquid chromatography connected with mass spectrometry to analyse anabasine and anatabine in urine [73,97,101–103]. However, the inconvenient of this technique is the instrument size, cost, difficulty of operation, and excessive heat and noise generation within the laboratory environment [104].

An alternative to traditional mass spectrometers is a mass detector, such as the Acquity QDa from Waters (Milford, USA). This detector is as intuitive as an optical detector because it is pre-optimised and sample-specific or user adjustments are not needed [105]. The ionisation mode consists in an ESI and the mass analyser – responsible for selecting ions based on their mass-to-charge ratio – is a quadrupole (Figure 9). The quadrupole allows the detection over a mass range of 30-1250 daltons and its size is 66% the size of the quadrupole in a traditional mass spectrometer [106]. The “miniaturisation” of components instrument and of the instrument itself is another advantage: it is easy to integrate to a HPLC system, using less bench space and less floor space. The ease of use and integration as well as the cost advantage make the QDa detector a good alternative for laboratories that need to benefit from the specificity of the mass spectral information [105]. However, few methods of quantification using a QDa detector are reported in the literature. Especially, there is no proposed method for the quantification of tobacco alkaloids.

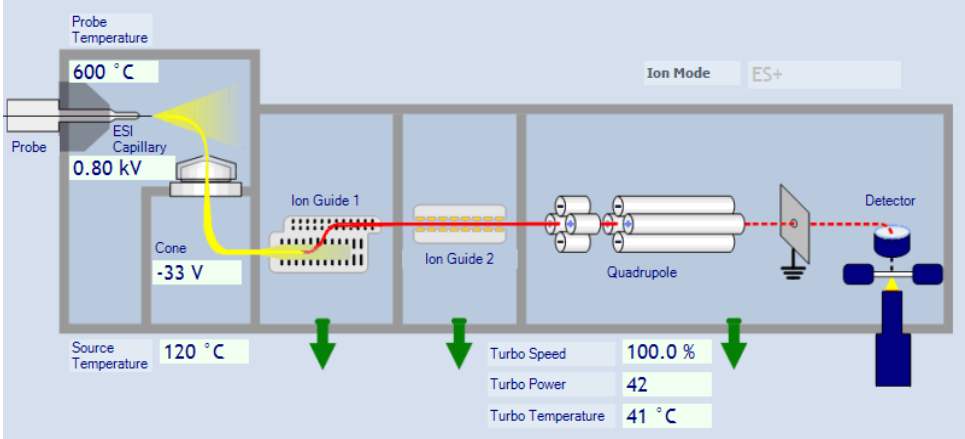


Figure 9: composition of the Acquity QDa detector. The liquid containing the compounds of interest is dispersed into a fine aerosol of charged droplets by electrospray ionisation (ESI). After solvent evaporation and droplet fission, gas-phase ions are produced. They reach the quadrupole that selects ions based on their mass-to-charge ratio. The selected ions are then detected [106].

5. Vascular function

5.1 Oxidative stress

5.1.1 Aerobic respiration

During aerobic respiration, human cells produce energy by converting oxygen to water [107]. This process leads to the production of intermediates, called reactive oxygen species (ROS), including superoxide anion, hydroxyl radical, and hydrogen peroxide (Figure 10). ROS are important because they play a regulatory role in cellular function under homeostatic conditions i.e. in case of equilibrium between ROS presence and the action of antioxidant defenses. Conversely, due to their highly biologically reactive properties, ROS in excess in case of "oxidative stress" can lead to various disease states by interacting with proteins, lipids, and DNA [108].

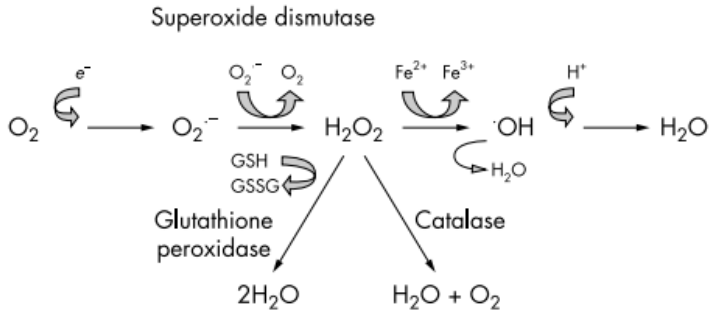


Figure 10: production of reactive oxygen species during aerobic respiration in human cells. Oxygen (O_2) reacts with an impaired electron (e^-) to generate a superoxide anion ($O_2^{\cdot-}$). The enzyme superoxide dismutase converts superoxide to hydrogen peroxide (H_2O_2). Hydrogen peroxide is converted to water (H_2O) via either the highly reactive intermediate hydroxyl radical ($^{\cdot}OH$) or the enzymes glutathione peroxidase or catalase [108].

5.1.2 Reactive oxygen species sources

ROS are generated by several enzymatic and non-enzymatic systems in blood vessels. It appears that NAD(P)H oxidases are major sources of ROS, particularly of superoxide, in vascular cells and myocytes. The NAD(P)H oxidases are membrane-associated enzymes catalysing the reduction of molecular oxygen in superoxide with NADH or NADPH as the

electron donor. The upregulation of vascular NAD(P)H oxidases activity, and therefore of superoxide production, by cytokines and hormones is related to the pathogenesis of several vascular diseases [108,109].

Vascular superoxide is also generated by xanthine oxidoreductase during the oxidation of hypoxanthine to xanthine in purine metabolism. Another source of vascular ROS production is the uncoupling of the endothelial nitric oxide synthase (eNOS). This point will be addressed further (point 5.3.1). Additional ROS sources incompletely understood are mitochondrial respiration, cyclooxygenases, lipoxygenases, and cytochrome P450 mono-oxygenase [108].

ROS that accumulate inside cells can also arise from an exogenous source. In this case, they mainly derive from inhaled toxic gases, such as environmental pollutants, car exhaust fumes, and tobacco smoke [110].

5.1.3 Reactive oxygen species role

ROS were first thought to be only deleterious and responsible for tissue injuries. But in the last few years, it was shown that ROS were signaling molecules at concentrations compatible with the cell physiology and are involved in modulation of cell viability and function. However, their role in cell signaling is not yet well understood because they are small and do not have structural and conformational complexity as traditional signaling molecules. Moreover, they interact very rapidly and within very short distances with the first organic molecule they encounter randomly. This characteristic makes difficult to consider ROS as molecules implied in regulation mechanisms [111].

5.1.4 Oxidant-antioxidant balance

The biological activity of ROS depends on their cellular concentration, which is regulated by the presence of antioxidant systems. These systems play a defensive role and can be enzymatic or nonenzymatic, endogenous or provided by the diet [108,110]. A first endogenous enzyme is the superoxide dismutase (SOD), which catalyses the metabolism of superoxide to hydrogen peroxide. Hydrogen peroxide is then detoxified to water by

combination with transition metal ions to form hydroxyl radical intermediate, or by glutathione peroxidase (GPX) or catalase (Figure 10) [108]. Nonenzymatic systems include small antioxidant molecules (carotenoids, vitamins E and C, glutathione, uric acid, lipoic acid, coenzyme Q_{10}, …) and proteins maintaining transition metals inactive for ROS formation (transferrin, ferritin, …) [108,110,112].

In case of oxidative stress, ROS level exceeds the one of antioxidants and ROS become toxic, leading to disease states. For example, they can alter proteins conformation by inducing the oxidation of sulfhydryl groups into disulfide bonds of cysteine residues. In this way, they cause modifications in enzymes activity or DNA binding. Moreover, they can be responsible for important cellular alterations through their interaction with ion channels and transcription factors. They induce lipid damage, an increase of cell permeability, uncontrolled cellular proliferation, and cell apoptosis or death. All these processes can in turn lead to a dysfunction of the endothelium, inflammation, and vascular remodeling and be the cause of cardiovascular diseases [110,113].

5.1.5 Tobacco smoke components involved in oxidative stress

Among the 5000 smoke constituents, there are organic compounds or metal ions acting as electrophiles; free radicals, reactive anions or metal ions acting as reducing agents (donate an electron); and free radicals or metal ions acting as oxidising agents (accept an electron). ROS and reactive nitrogen species (RNS) are produced when the mainstream cigarette smoke interacts with aqueous media or physiological fluids [17,114]. Some component become involved in oxidative stress only after chemical modification by metabolic processes *in vivo*. For example, benzo[a]pyrene is first metabolised in quinone, which can in turn generate ROS via a redox cycling mechanism [115]. Some oxidants are generated in response to inflammation resulting from smoking-related oxidative stress [17].

5.1.5.1 Particulate phase constituents and oxidative stress

Three types of molecules of the smoke particulate phase are implied in the onset of oxidative stress: free radicals, quinones and trace heavy metals.

Free radicals

The tar fraction of cigarette smoke contains very high concentrations of stable free radicals (10^{17} per gram) with very long lifetimes [116].

A mechanism proposed for the formation of superoxide found in the aqueous extract of cigarette tar (ACT) is the autooxidation of hydroquinone (QH_2) – a tobacco smoke polyphenolic agent – in air to form benzosemiquinone radical ($QH^{\cdot}$). The $QH^{\cdot}$ radical reduces O_2 into superoxide ($O_2^{\cdot-}$), which can dismutase to form H_2O_2. Then, H_2O_2 and Fe^{2+} – contained in high concentration in tar – produce highly oxidising hydroxyl radicals ($^{\cdot}OH$) through the Fenton reaction [17,116,117].

$$Q + QH_2 \leftrightarrows 2QH^{\cdot}$$

$$QH^{\cdot} + O_2 \rightarrow Q + O_2^{\cdot-} + H^+$$

$$O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^- \textit{ (Fenton reaction)}$$

Hydrogen peroxide is naturally produced during the respiration process by catalytic disproportionation of superoxide radicals by superoxide dismutase (Figure 10). Another enzyme, catalase, converts hydrogen peroxide to water and molecular oxygen. However, when this cellular defense mechanism is overwhelmed, the excess H_2O_2 can undergo the Fenton reaction and forms hydroxyl radicals. These highly oxidising species are well known to cause oxidative damage to essential biomolecules, such as DNA. H_2O_2 has also been found in ACT and in aged smoke. Therefore, hydrogen peroxide can be exogenous and found in tobacco smoke or it can be formed from the physiological response to smoke constituents presumed to be a source of oxidative stress [17,117].

Quinones

Quinones – derived from tobacco smoke constituents that undergo autooxidation – are toxic and involved in oxidative stress by two mechanisms: (i) the redox cycling mechanism

which produces excess ROS as byproducts, and (ii) the formation of covalent bonds with essential biomolecules, especially those containing thiol groups [118,119].

Quinones are reduced to hydroquinones or semiquinone radicals through the NADPH pathway (Figure 11). Both hydroquinone and semiquinone radical undergo autooxidation to regenerate the parent quinone, thereby creating the redox cycle associated with ROS – superoxide and H_2O_2 – production. It increases the cellular ROS level and depletes antioxidant defenses [17,120].

Quinones react readily with cellular nucleophiles such as thiols, impairing cellular function. An example is the conjugation of quinones with glutathione (GSH), whose the main purpose is to protect cells from reactive electrophiles and free radicals. Moreover, GSH can also be oxidised to GSSG by the hydroxyl radicals produced during the redox cycling of quinone. These two processes cause GSH depletion [17,120,121].

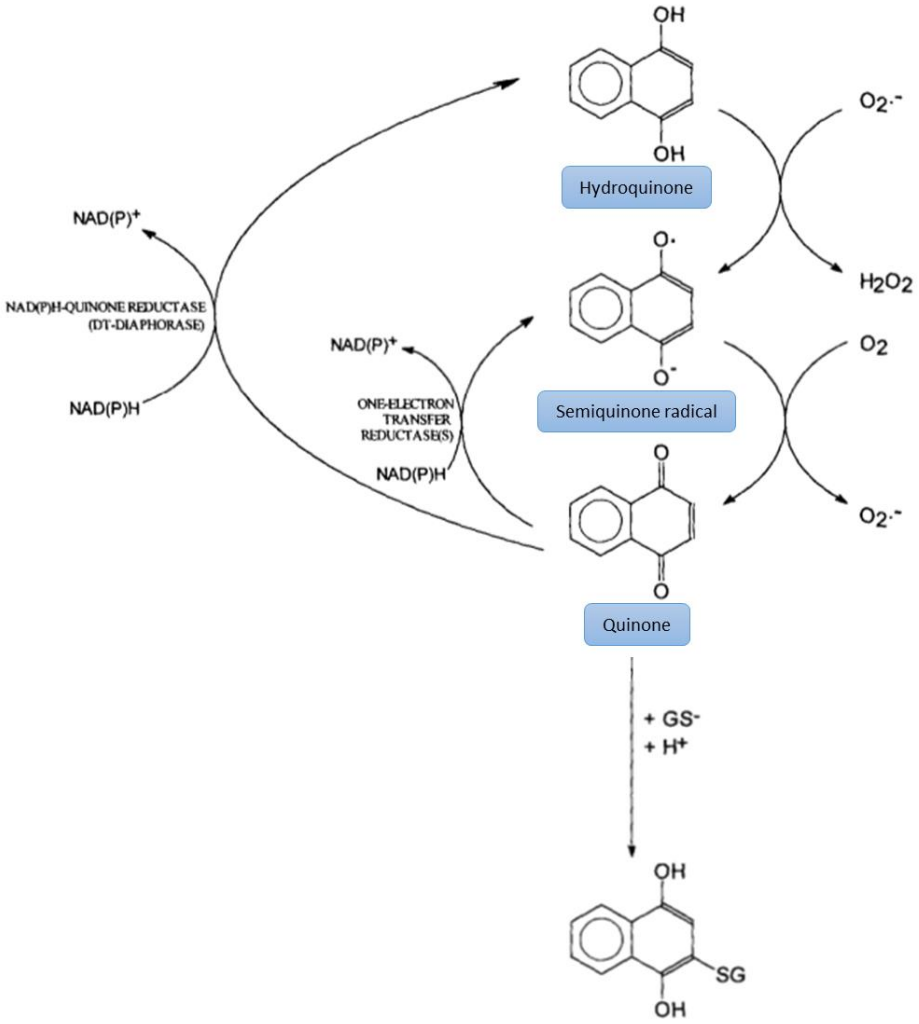


Figure 11: the two mechanisms of quinone-induced stress oxidative. (i) Quinones are reduced to hydroquinones or semiquinone radicals, involving the NADPH pathway. The semiquinone radical undergoes rapid autooxidation to regenerate the parent quinone, thereby producing superoxide. The hydroquinone reacts with superoxide to form H_2O_2 and the semiquinone; (ii) Most quinones also react with cellular nucleophile, such as thiol of glutathione (GSH) [120].

Trace heavy metals

Given that tobacco plants transport metal ions from the soil into the leaves, trace amounts of heavy metals are found in the cigarette smoke. Among these metals, some are redox-inactive – cadmium, lead, mercury, and arsenic – or redox-active – copper, iron, nickel, and

chromium [122,123]. The first ones lead to oxidative stress by depleting cells of thiol-containing antioxidants and reducing antioxidant enzymes activity. The others can undergo redox cycling in oxygenated aqueous solutions and thereby generate ROS. Heavy metals can have other molecular effects, including inhibition of DNA repair and activation of cellular signaling [124–127].

Transition metals contained in the tar of tobacco smoke, such as iron (Fe^{2+}), can promote the formation of hydroxyl radicals through the Fenton reaction [128,129]. Cu^{+} is also known to be active in hydroxyl radicals formation. Moreover, Cu^{2+} oxidises catechol and hydroquinone to their respective quinones and is, therefore, involved in the redox cycle that forms semiquinones radicals and generates superoxide [130].

5.1.5.2 Gaseous phase constituents and oxidative stress

ROS are found in the cigarette gaseous phase and are known to promote the destruction of endogenous antioxidant defenses [116].

Oxidising radicals and reactive nitrogen species

Many oxidising agents are found in the gaseous phase of cigarette smoke and are all short-lived. There are about 10^{15} free radicals per puff in this phase. Nitric oxide ($NO^{\cdot}$) is a radical of interest because of its multiple physiological roles. This radical is not particularly reactive nor toxic but it leads to toxic effect when generated in excess [17,116].

$NO^{\cdot}$ reacts with molecular oxygen in air to form $NO_2^{\cdot}$ – a toxic oxidant and nitrating agent. $NO_2^{\cdot}$ in turn reacts with other smoke components, such as isoprene and butadiene, to form highly reactive nitrosocarbon-centered radicals. These carbon-centered radicals react instantaneously with molecular oxygen to form peroxyl radicals, which react with $NO^{\cdot}$ to generate alkoxyl radicals and $NO_2^{\cdot}$. It creates, thereby, a continuous cycle. Therefore, these oxidising radicals found in the gaseous phase are formed by reactions between the phase constituents and not by the pyrolysis or combustions processes. The radicals level in the gaseous phase increases until the supply of $NO^{\cdot}$ is depleted and then persists for several minutes [131]. However, the involvement of these free radicals in damage caused by

oxidative stress is still unclear given that radicals are generally believed to be quenched immediately on contact with surfaces of the respiratory tract [132].

$NO^{\cdot}$ can also react with tyrosyl radical, which is present at the active sites of some enzymes, such as ribonucleotide reductase [133]. $NO^{\cdot}$ may be converted to more reactive derivatives, such as $NO_2^{\cdot}$, N_2O_3, N_2O_4 and peroxynitrite ($ONOO^-$) – called "reactive nitrogen species" (RNS). RNS are responsible for DNA damage and nitration of tyrosine in cells exposed to the gaseous phase of tobacco smoke [134,135]. Moreover, $NO^{\cdot}$ is known to stimulate the toxicity of phenolic compounds by oxidation to their respective quinones [17,136].

Peroxynitrite

The reaction between the two free radicals $NO^{\cdot}$ and superoxide forms peroxynitrite ($ONOO^-$), a powerful oxidant that induces damage to essential biomolecules. There is a competition between $NO^{\cdot}$ and SOD for reacting with superoxide. The toxicity of superoxide is mostly due to the formation of peroxynitrite [116,137,138].

To generate peroxynitrite, it is generally believed that $NO^{\cdot}$ arising from the cigarette gaseous phase reacts with superoxide derived from the reducing constituents of the particulate phase, such as hydroquinone and catechol [139]. Peroxynitrite then combines with abundant intracellular carbon dioxide to form nitrating, nitrosating, and oxidising intermediates. It can also react with essential proteins, including haemoglobin, myeloperoxidase, GPX, and others, resulting in their inactivation [134,140].

Glutathione depleting substances

Glutathione (GSH) is found in abundance in cytoplasm, nuclei, and mitochondria and is the main water-soluble antioxidant in these cell compartments [141]. The gaseous phase of smoke contains high levels of various reactive aldehydes which can react with thiol of GSH to form potentially toxic GSH-aldehyde adducts. For example, acrolein and crotonaldehyde – two α,β-unsaturated aldehydes found in cigarette mainstream smoke – can react with thiol of GSH or with thiol-containing proteins, resulting in their inactivation [142]. It was shown that the exposition to the gaseous phase of cigarette smoke resulted in a significant

depletion of GSH and also in the appearance of oxidised GSH (GSSG). However, the depletion of GSH due to the formation of GSSG accounts for only 25% [17,143].

5.2 Endothelial function

5.2.1 Vascular wall components

Three layers compose the blood vessels: the endothelium, the medial vascular smooth muscle and the adventitia (Figure 12) [144].

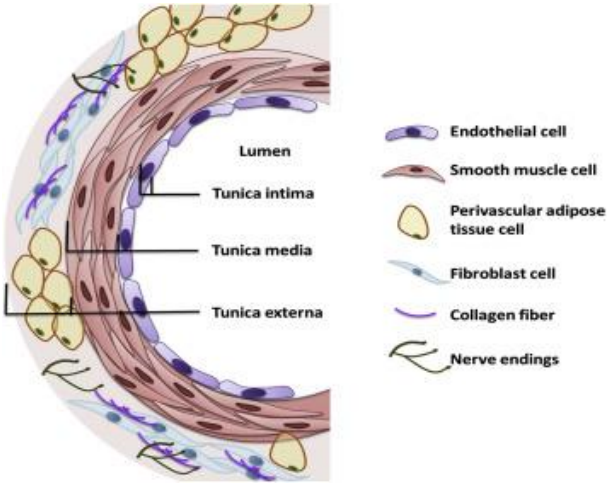


Figure 12: arterial wall structure. The arterial wall is composed of three layers: (i) tunica intima (endothelial cells and internal elastic membrane), (ii) tunica media (vascular smooth muscle cells and external elastic membrane), and (iii) tunica externa (adventitia containing perivascular adipose tissue cells, fibroblast cells, collagen fibers and nerve endings) [144].

Endothelial cells form an intimal monolayer – the endothelium – separating the lumen of the blood vessels and the surrounding tissues. This barrier plays an important role in vascular homeostasis. Its other functions are the regulation of vascular tone, the maintenance of blood fluidity, and the control of inflammation and immune response [144,145].

The medial vascular smooth muscle allows the constriction and dilatation of the blood vessels following a mechanical or pharmacological activation [144].

The adventitia contains fibroblasts and collagen fibers allowing the connection with surrounding organs. This layer is also composed of adipose tissue and nerve endings and is involved in signal exchanges between the blood vessels and the tissues, including inflammatory cell trafficking [144,146].

5.2.2 Endothelium functions

The endothelium can be considered an autocrine and paracrine organ which produces chemical mediators, growth factors, and vasoactive molecules. It plays, therefore, a major role in maintaining the vascular homeostasis [147].

Endothelial cells insure a proper homeostatic balance between pro- and anticoagulant mechanisms allowing to prevent bleeding or thrombosis. They release components, such as P-selectin, which mediate platelet and leukocytes adhesion before their migration into inflamed tissue. These cells also produce growth factors implied in blood vessel formation as well as quiescence and stability of the mature vasculature maintaining [148].

Another important role of endothelial cells is the regulation of the vascular tone by secreting dilating agents – nitric oxide (NO), prostacyclin, bradykinin, and endothelium-derived hyperpolarising factor (EDHF) – or constricting substances – endothelin, superoxide anion, angiotensin II, and thromboxane [149,150].

The action of NO – an endothelium-derived relaxing factor (EDRF) – was first described. This gas is produced from L-arginine and requires the activity of the endothelial nitric oxide synthase (eNOS), an enzyme present in endothelial cells [149,150]. NO is continuously secreted by healthy endothelial cells [149]. It was shown that a decrease of its bioavailability leads inevitably to endothelial dysfunction [151].

The modulation of the vascular tone can also be insured by an NO-independent pathway that appears important in case of a decrease of NO bioavailability. The endothelium produces EDHF – not well-characterised – increasing potassium conductance and inducing subsequent relaxation of smooth muscle cells (SMC) [149–151].

5.2.3 Endothelial nitric oxide

5.2.3.1 NO biological actions

NO plays diverse roles in endothelial function. Diffusion of NO from the endothelium into smooth muscle is responsible for its vasodilation through the activation of guanylate cyclase, leading to an increase of intracellular cyclic guanine monophosphate (cGMP). NO diffusing in the other direction, from the endothelium into the lumen, is captured by circulating platelets, inducing the inhibition of platelet adhesion and aggregation [149]. An insufficient NO production results in increased cell adhesion molecule expression, such as P-selectin, and thus in increased adherence of leukocytes and platelets to the endothelium [152]. NO also prevents the migration of leukocytes into vascular tissue by the monocyte chemoattractant protein 1 (MCP-1) expression inhibition, an important chemoattractant protein [149].

5.2.3.2 Regulation of NO biosynthesis

The endothelial nitric oxide synthase (eNOS) – the enzyme responsible for NO biosynthesis – is regulated in the short and in the long term through different ways. The main regulation in the short term (occuring within seconds) is the eNOS activation by calcium stimuli, including all reactions inducing an intracellular free calcium levels increase, such as shear stress. Others are intracellular levels of tetrahydrobiopterin (BH_4), a cofactor of eNOS, or NO itself acting as negative feedback regulator of its own production [144,149,153]. As for the eNOS long-term regulation, it is due to prolonged shear stress, cytokines, growth, and possibly estrogens [149,153].

5.3 Endothelial dysfunction

5.3.1 Causes and mechanisms

Endothelial dysfunction, the hallmark of several cardiovascular diseases, is defined by the loss or dysregulation of the homeostatic mechanisms normally occurring in healthy endothelial cells. It is associated with reduced vasodilation, a proinflammatory state, and prothrombic properties and may lead to cardiovascular diseases, such hypertension, chronic heart failure, thrombosis, etc. [148,150,154–156]. This state is characterised by the activation of the endothelium due to ROS. They impair the NO balance and activate cellular processes that are normally silenced by the NO action, through in part the activation of NF-κB. NF-κB is a nuclear factor inducing the transcription of proinflammatory genes and the expression of adhesion molecules – E-selectin, vascular cell adhesion protein 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) [110,148,157]. Besides imbalance the NO levels, ROS can damage the endothelium and make it permeable to cells supposed to remain in blood. These ones can pass through blood vessels and reach adjacent tissues, leading to an inflammatory state [148]. All these processes result in expression of chemokines, cytokines, and adhesion molecules that interact with leukocytes and platelets and target inflammation. ROS are produced from oxidases but also from the uncoupled state of eNOS. Thus, eNOS can switch between maintaining the quiescent state of the endothelium and generating ROS in case of endothelial activation (Figure 13) [150]. This alteration is called "eNOS uncoupling" and causes the production of: (i) superoxide ($O_2^{\cdot-}$) if the cofactor BH_4 is absent, (ii) hydrogen peroxide (H_2O_2) in case of the substrate L-arginine deficiency [150,158].

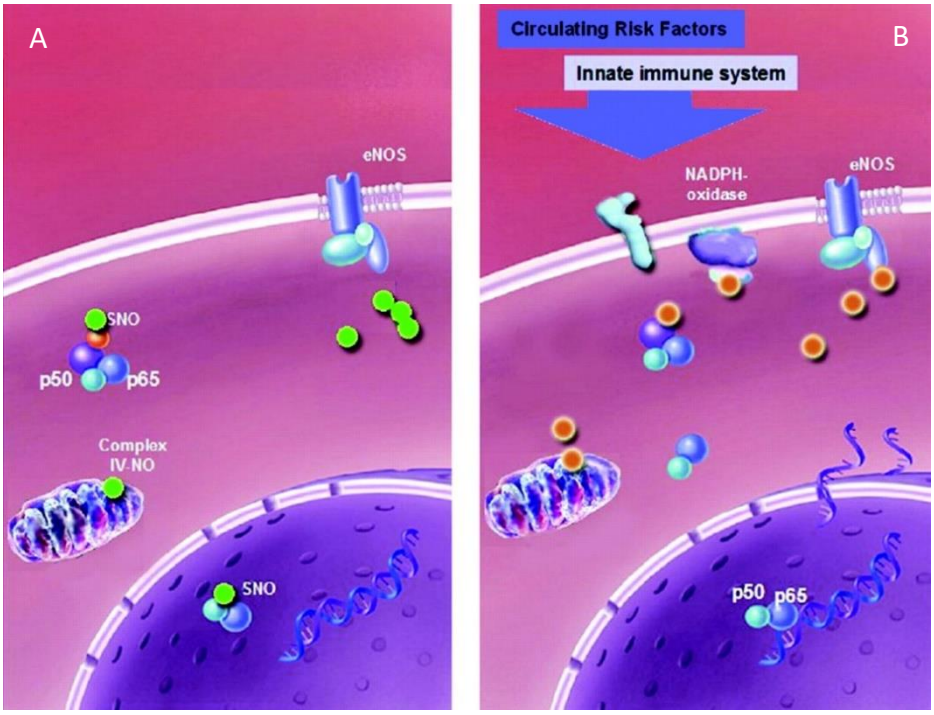


Figure 13: the quiescent state of the endothelium (A) and the state of endothelial activation (B). (A) Nitric oxide (NO) (green circles) is produced by endothelial nitric oxide synthase (eNOS) and targets cysteine groups of key regulator molecules, such as NF-κB (subunits p50/p65), and the mitochondria. It leads to cellular processes silencing. (B) Reactive oxygen species (ROS) (orange circles) predominate and cause the activation of the endothelium. ROS are produced by oxidases and the uncoupled eNOS. They also target key regulator proteins, such as NF-κB and phosphatases. The endothelial activation can occur in host defense context or in the presence of cardiovascular risk factors [150].

5.3.2 Atherosclerotic process

Atherosclerosis – considered an inflammatory autoimmune disease – is characterised by specific histopathological changes in the arterial wall and begins with endothelial dysfunction. This state promotes the accumulation of lipids, fibrous tissue, and macrophages, leading to the formation of atherosclerotic plaques [110,159].

ROS and peroxynitrite ($ONOO^-$) – generated from the reaction between NO and superoxide produced by the uncoupled eNOS – induce low-density lipoprotein (LDL) oxidation in arteries [110,155]. Oxidised LDL (oxLDL) play a crucial role in the development of

atherosclerosis, being an important proinflammatory stimulus for chronic inflammation. They activate the endothelium and promote the expression of adhesion molecules and the secretion of chemokines that are involved in the recruitment of circulating leukocytes – macrophages – into the endothelium (Figure 14). These ones internalise oxLDL and form the "foam cells" implied in the secretion of inflammatory mediators [154]. Foam cells contribute to the formation of atherosclerotic plaques which can lead to thrombosis in case of rupture [159–161].

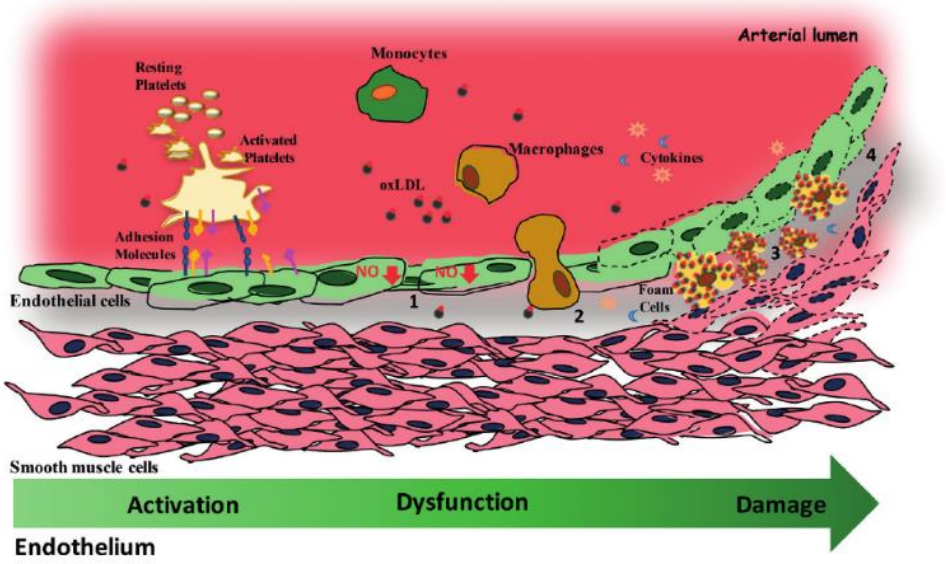


Figure 14: signaling pathways involved in endothelial dysfunction and atherosclerosis. Endothelium is activated due to oxidative stress, inducing the expression of adhesion molecules and the activation of macrophages and platelets. NO level is reduced, activated platelets adhere to endothelium, macrophages migrate through the endothelium and uptake oxidised low-density lipoprotein (oxLDL) to form foam cells and thus the atherosclerotic plaque [110].

6. Effects of smoking on endothelial function

6.1 Impact of the tobacco smoke on cardiovascular diseases

Tobacco smoking is a major preventable risk factor for the development and progression of cardiovascular diseases (CVDs) [26,162]. Approximately 35-40% of smoking-related deaths are due to CVDs. It was shown that active smoking was not the only one involved in morbidity and mortality related to CVDs, secondhand smoke contributes also to them [163,164]. Tobacco smoking is associated with many pathological conditions leading to atherothrombosis, such as endothelial dysfunction, inflammation, insulin resistance, dyslipidaemia, haemodynamic alterations, and hypercoagulability. However, the mechanisms underlying the link between smoking and CVDs are not yet completely understood [11,165,166].

6.2 Role of tobacco smoke components in cardiovascular diseases

Tobacco smoking as well as the use of smokeless tobacco products impair eNOS-dependent arterial dilation. Nevertheless, the precise tobacco component responsible for vascular dysfunction remains unknown [167]. Nicotine, carbon monoxide (CO), and oxidant gases are the most studied constituents of tobacco smoke as potential players in CVDs development [11].

Nicotine seems to impact the cardiovascular system by stimulating sympathetic neural system. It leads to haemodynamic effects, such as an increase of heart rate, blood pressure, and cardiac output, and implies, therefore, an increase in myocardial oxygen demand. It is still unclear whether nicotine plays a direct role in CVDs development. At present, nicotine has been reported to have variable effects on NO *in vitro* and could, in this way, contribute to endothelial dysfunction in tobacco users [11,110,167].

Inhalation of CO from tobacco smoke was first thought to be the cause of cardiovascular effects. However, it appears that CO is not involved in atherothrombosis process associated with cigarette smoking. Data showed that inhaled CO, delivered to healthy people in

concentrations similar to those in cigarette smoke, does not affect heart rate, blood pressure, catecholamine secretion, platelet activation, or serum C-reactive protein (CRP), an inflammatory biomarker [11,110,168].

Since oxidative stress plays a major role in the development of atherosclerosis, oxidant gases could be the prime causative agents of endothelial dysfunction in smokers. Cigarette smoke contains many oxidising chemicals, including NO and free radicals. These free radicals could arise from: (i) the vapor or particulate phases of cigarette smoke, (ii) circulating or in situ-activated macrophages and neutrophils, (iii) platelets, which their activation can induce superoxide production, and/or (iv) endogenous systems, such as uncoupled eNOS, xanthine oxidase, and the mitochondrial electron transport chain [25]. In addition, smokers are known to have lower antioxidant defenses as, for example, vitamin C and β-carotene [169,170]. Therefore, an antioxidants supplementation such as vitamin C could improve the smokers' endothelial function [171].

Other components of cigarette are involved in endothelial dysfunction. Large number of metals, including aluminum, cadmium, copper, lead, mercury, nickel, and zinc, are present in cigarette smoke. They contribute to the oxidation of cellular proteins, causing structural cellular damages and endothelial dysfunction [11]. Apolipoprotein A-1 – the main protein in high-density lipoprotein (HDL) – is modified by acrolein contained in high levels in cigarette smoke, leading to the oxidation of thioredoxins which are antioxidant proteins in endothelial cells [172]. This modification causes endothelial cells death and dysfunction [173]. Finally, polycyclic aromatic hydrocarbons present in the tar fraction of cigarette are known to contribute to the atherosclerosis process [174].

6.3 Mechanisms and biomarkers involved in tobacco-related endothelial dysfunction

Several pathophysiologic mechanisms are involved in endothelial dysfunction and the development and progression of atherothrombosis (Figure 15). Endothelial dysfunction seems to be mostly correlated with oxidative stress and inflammation and all these processes are closely related to each other [110].

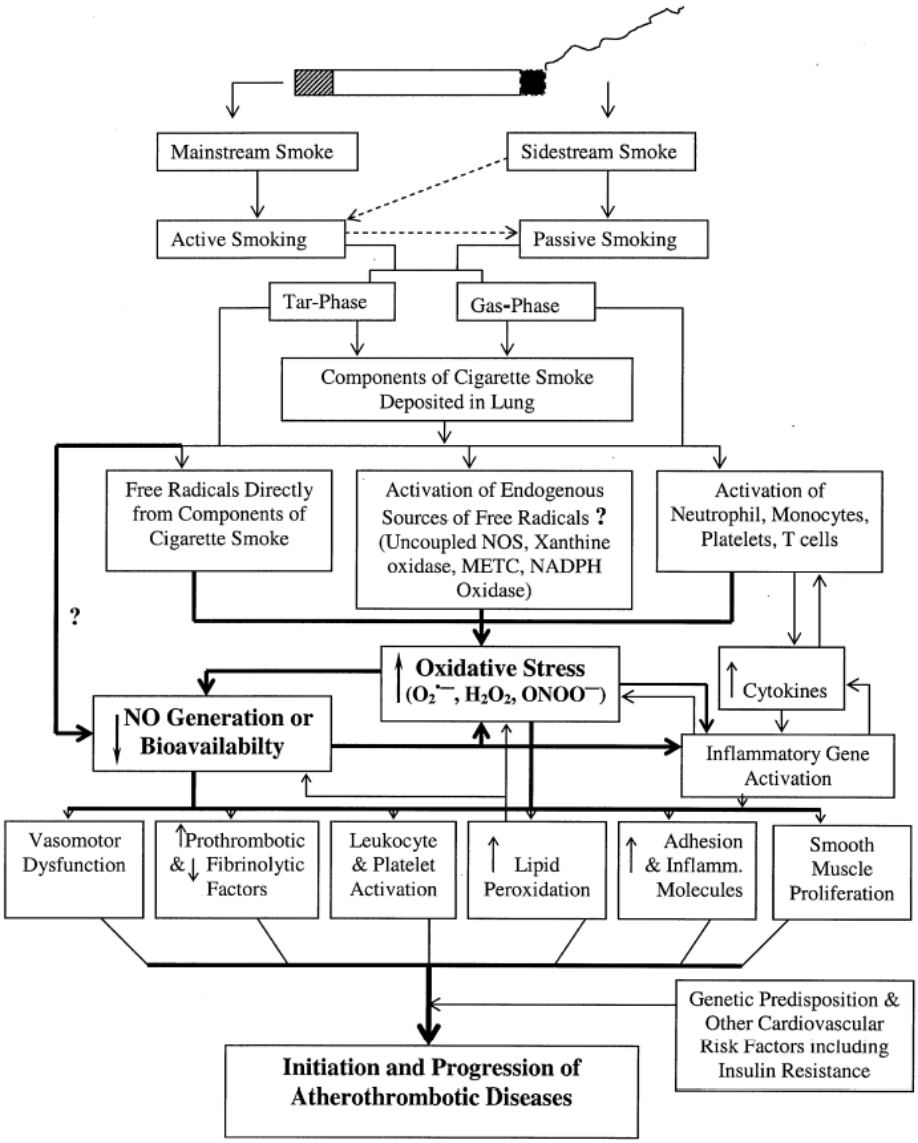


Figure 15: potential mechanisms involved in cigarette smoking-mediated cardiovascular dysfunction. The bold boxes and bold arrows show the probable central mechanisms in the pathophysiology of cigarette smoking-mediated atherothrombosis.
NOS = nitric oxide synthase; METC = mitochondrial electron transport chain; NADPH = nicotinamide adenine dinucleotide phosphate reduced form; $O_2^{\bullet-}$ = superoxide; H_2O_2 = hydrogen peroxide; $ONOO^-$ = peroxinitrite [25].

6.3.1 Vascular and endothelial dysfunction

Tobacco smoke exposure impairs endothelium-dependent vasodilation (EDV) in macrovascular beds, such as coronary and brachial arteries, and in microvascular beds. The decrease of NO availability – the prime responsible for the endothelium vasodilatory function – is mainly associated with this effect [11,25]. Clinical evaluation of endothelial dysfunction can be performed by measuring the NO bioavailability levels or the flow-mediated dilation (FMD). The FMD is evaluated by measuring target artery diameter by high-resolution external vascular ultrasound following a blood flow increase induced by cuff inflation and then deflation. The advantage of this test is its noninvasive nature [110,175]. It was demonstrated that FMD was compromised in smokers but that smoking cessation can improve this parameter [110,176]. Asymmetric dimethylarginine (ADMA), an endogenous analogue of L-arginine, could also be used as a biomarker reporting a reduction in the NO bioavailability. High level of ADMA competitively inhibits the eNOS and thus results in a decrease of the NO production and in endothelial dysfunction [177,178].

Another effect of smoking is damages of the vascular wall resulting in impaired prostacyclin production and an increase of platelet-injured vessel wall interactions [11,110].

Finally, endothelial dysfunction is associated with the increased ability of endothelial cells to adhere to immunity cells, such as monocytes, macrophages, T lymphocytes, and platelets. Levels of adhesion molecules such as VCAM-1, ICAM-1, E-selectin and P-selectin can be measured as endothelial function biomarkers. Many studies reported higher concentrations in smokers' plasma than in non-smokers' plasma [25,110,179].

6.3.2 Oxidative stress

A consequence of smoke compounds exposure is the ROS overproduction resulting in endothelial dysfunction as well as endothelial cell loss by apoptosis or necrosis [180]. As previously mentioned, ROS can arise from exogenous or endogenous sources. The main exogenous ROS source is inhaled toxic gases, such as tobacco smoke. Tobacco smoke

contains free radicals and oxidative gases which can induce ROS intracellular production and reduce intracellular antioxidant mechanisms, resulting in oxidative stress [110].

Oxidative stress can be evaluated by many parameters, such as changes in antioxidant defense system status, lipid peroxidation, and protein oxidation. Some of them were shown affected in smokers [110,181].

Concentration of enzymes involved in antioxidant mechanisms, such as GPX, SOD, and catalase, can be quantified. Other antioxidant systems can be measured, for example carotenoids, vitamins C, A, and E, glutathione, uric acid, coenzyme Q_{10}, transferrin, ferritin, … Some studies reported that vitamin C and vitamin E levels were significantly lower in smoker group than in non-smoker group. Others indicated a decrease of GPX and SOD activities after cigarette smoke exposure [110,112,182]. Conversely, prooxidant molecules can also be quantified, such as iron which is prone to generate hydroxyl radical by reacting with hydrogen peroxide in the Fenton reaction and cause oxidative cell damage [183].

Malondialdehyde (MDA), 4-hydroxy-nonenal (HNE) and F2-isoprostane 8-iso-prostaglandin F2α (8-iso-PGF2α) are the main investigated products of lipid peroxidation and are the most frequently measured biomarkers of this process [181,184]. It is also possible to directly measure lipid peroxides in plasma [185,186]. Data showed that cigarette smoke induces a significant increase of MDA and 8-iso-PGF2α, indicating an increased lipid peroxidation [25,182,187].

Cigarette smoking enhances oxidative modification of LDL which forms the basis of foam cells and leads to atherosclerosis. Besides an increase of circulating products of lipid peroxidation, an increase of anti-oxidised LDL antibodies level is reported in smokers [25]. It has also been suggested that smoke stimulates SMC proliferation and migration, leading to a thickening of the intima and plaque formation. Another consequence of cigarette smoke exposure is the death of SMC by necrosis, causing the activation of inflammatory signals (point 6.3.3) [110].

When proteins are oxidised by ROS, carbonyl groups – aldehydes and ketones – are produced on their side chains, especially of proline, arginine, lysine, and threonine. Protein carbonyl content is the most commonly used marker of protein oxidation and is higher in smokers compared to non-smokers [187–189].

6.3.3 Inflammation

Oxidative stress and inflammation are closely related: the overproduction of ROS promotes the expression of inflammatory genes while the activation of immune cells, such as macrophages or neutrophils, causes free radicals generation. These two processes lead to each other [110].

Inflammatory process is a major component in the initiation and evolution of endothelial dysfunction and atherosclerosis. There is a lot of evidence that associate cigarette smoking with chronic inflammation and this link can be reflected by many inflammatory markers [11,25].

Tobacco smoking, and in particular the number of cigarettes smoked daily, is strongly correlated with blood leukocyte count. Studies showed a 20-25% increase in the peripheral blood leukocyte count in smokers [11,166,190].

The extent of systemic inflammation can be evaluated by measuring the plasma/serum level of C-reactive protein (CRP). A study showed that lifetime exposure to smoking is strongly associated with CRP, even in people who have stopped smoking. It suggests that some effects of smoking remain for many years after cessation [191]. Another one reported significantly increased levels of circulating total white blood cells, lymphocytes, monocytes, neutrophils, basophils, and CRP in smokers compared with never-users of tobacco products [192].

Measurement of pro- and anti- inflammatory cytokines in serum can also be used as informative inflammatory markers [193]. Local (vascular) or systemic (extravascular) inflammation triggers the production of primary proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [194,195]. These primary

proinflammatory cytokines and oxLDL in turn activate the endothelium and the expression of adhesion molecules [196,197], as previously mentioned (point 6.3.1). Moreover, primary proinflammatory cytokines induce the production of chemoattractant cytokines (chemokines) which appear to play a major role in atherogenesis by inducing chemoattraction of monocytes and activated T lymphocytes. Chemokines include MCP-1 – expressed by macrophages, SMC, and endothelial cells – and interleukin-8 (IL-8) – expressed mainly by macrophages in human lesions [194]. Finally, primary proinflammatory cytokines stimulate the generation of interleukin-6 (IL-6), a secondary proinflammatory cytokine, which subsequently induces the production of acute-phase proteins by the liver, such as CRP, serum amyloid A, and fibrinogen [198]. Interleukin-10 (IL-10) is an anti-inflammatory cytokine produced by macrophage and T lymphocytes which has an anti-atherogenic effect by inhibiting macrophage activation. Through the inhibition of NF-κB, IL-10 prevents the expression of inflammatory mediators, such as TNF-α, MCP-1, and ICAM-1 [199]. Studies showed an increase in the IL-6 and TNF-α expression and a decrease of IL-10 level in smokers compared to non-smokers [25,110,200].

OBJECTIVES

It is now conclusive that smoking is a risk factor for the development of several diseases, especially of cardiovascular diseases (CVDs). Although the implied mechanisms are still unclear, it appears that smoking induces several processes involved in CVDs: endothelial dysfunction, oxidative stress, inflammation, and altered lipid profile. The tobacco component(s) implicated is (are) not yet identified. In this context, the potential role of nicotine in CVDs remains to be elucidated. It is especially of interest in view of the emergence of non-tobacco nicotine delivery products, such as electronic nicotine delivery devices or nicotine containing medications used in smoking cessation. The main goal of this thesis was to evaluate the effect of tobacco but also of nicotine replacement therapy (NRT), prescribed for smoking cessation, on the endothelial function. To that end, biomarkers reflecting the endothelial function were measured in smokers and nicotine users in order to achieve an objective and non-invasive assessment. As endothelial dysfunction is linked to oxidative stress, inflammation, and dyslipidaemia, biomarkers of these processes were also studied. These measurements were evaluated according to the tobacco smoke or nicotine exposure, which was also objectified by biomarkers. To reach the global objective, the experimental work was split in four chapters.

The first chapter concerns the analytical part of the work. Based on the literature, we have selected a panel of biomarkers to reflect endothelial dysfunction and we had to be able to quantify them in participants' body fluids. Some ones were already routinely analysed in our medical laboratory, but other ones required the development of new analytical methods. The first developed method allowed the quantification of cotinine, the biomarker of choice for nicotine uptake. We then used the same strategy to develop a method to quantify simultaneously five tobacco biomarkers: nicotine, cotinine, trans-3'-hydroxycotinine, anatabine, and anabasine. This method was useful to discriminate between active smokers and nicotine users among participants. Finally, the third and the last development was performed to analyse malondialdehyde, a biomarker of lipid peroxidation occurring in case of oxidative stress.

The second chapter answers to two objectives. The first one was to evaluate the endothelial dysfunction in smokers in comparison with non-smokers. This strategy allowed us to

identify the implied processes. Moreover, we showed the impact of smoking on the endothelial dysfunction-related biomarkers, which was not yet studied for some of them. Data collected for smokers and non-smokers were then used for the second objective. We evaluated the possibility of discriminating smokers from non-smokers based on biomarkers other than nicotine and its metabolites. In this way, smokers, nicotine users and non-smokers could be identified. All studied biomarkers were assessed in this role and discriminating features were analysed.

In the third chapter, we aim to evaluate the effect of smoking cessation on endothelial dysfunction. We measured biomarkers concentrations in smokers at baseline and post-quit (median abstinence duration of 70 days) to show the potential changes. It allowed us to determine if smoking-induced endothelial dysfunction could be reversible and within what time frame.

Finally, the fourth chapter addresses the effect of NRT on endothelial dysfunction. This aid is effective for smoking cessation, but its safety concerning CVDs and endothelial function remains controversial. Therefore, its prescription could be restrained. We followed the biomarkers concentration changes in smokers on quitting who resorted to NRT treatment or not. The comparison of the two groups informed us about the impact of NRT on endothelial dysfunction.

POPULATION

The study participants were recruited at the tobaccology unit of the Centre Hospitalier Universitaire (CHU) of Catholic University of Louvain (UCL) (Namur, Belgium). We aimed to recruit seventy-five smokers and seventy-five non-smokers. The sample size was determined in order to be able to observe a medium effect (Cohen's d=0.5 [201]) with a power of 80% and an alpha (α) risk of 5%. Finally, one hundred and thirty-eight active cigarette smokers and eighty-three healthy long-term non-smokers were recruited (Figure 16). The potential presence of clinical pathology that may influence the endothelial function was studied through the analysis of confounding factors. Ten of smokers suffered from chronic obstructive pulmonary disease (COPD) without acute pathology. Informed consent was provided from all participants. The project was approved by the hospital ethics committee and was led according to the Declaration of Helsinki and the Good Clinical Practice guidelines [202].

Among smokers, sixty-five were followed during their cessation program. Thirty-five used Nicotine replacement therapy (NRT) products (gum, transdermal patches, oral sprays, or inhalers), 22 resorted to varenicline (Champix®), and 8 quit without medication. The median smoking abstinence duration was 70 days (interquartile range: 45 – 105 days), with a minimal duration of 21 days.

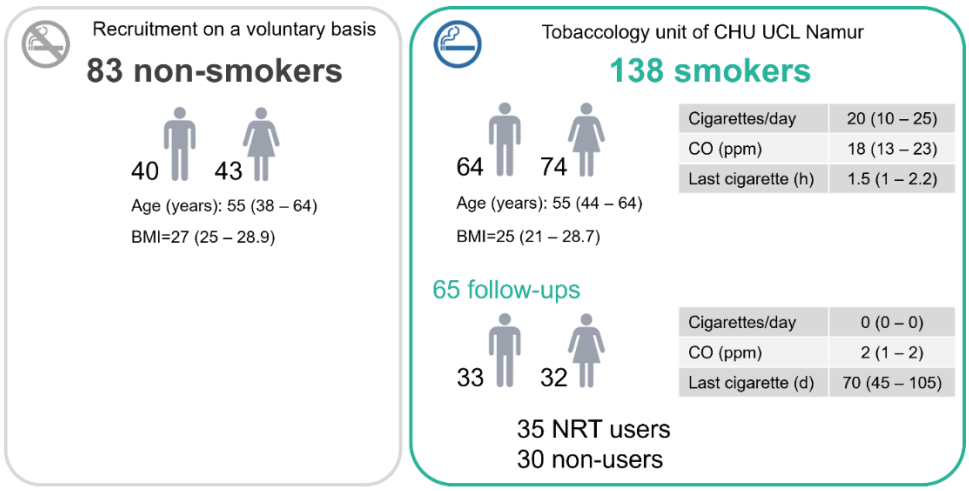


Figure 16: Features of the study participants. Data are expressed by median (interquartile range).

RESULTS AND DISCUSSION

Chapter I. Development and validation of new analytical methods

Tobacco use is a risk factor for endothelial dysfunction, the first step of cardiovascular diseases (CVDs) development [1,2]. Endothelial dysfunction is associated with oxidative stress, inflammation, and lipid profile modification [1–3]. The measurement of the extent of these processes linked to the tobacco exposure could objectively inform of the effect of smoking on CVDs and the implied mechanisms. Actually, the latters are not completely understood [1].

The evaluation of individuals' endothelial function, oxidative status, inflammatory status, lipid profile, and tobacco exposure can be performed by the measurement of various biomarkers in body fluids [4–7]. Suitable laboratory procedures are required to this end. Among the selected biomarkers for this project, some were routinely quantified in our medical laboratory while others needed the specific development of the analysis. These developments are addressed in this chapter.

The first developed analytical method concerned the evaluation of tobacco smoke exposure through the quantification of urinary cotinine. It is the main metabolite of nicotine and the most widely used biomarker of nicotine intake [8,9]. Its measurement was essential in this project to discriminate between nicotine users, from smoking or nicotine replacement therapy (NRT), and non-users [8,10]. This alkaloid was already analysed in our laboratory by ultra-high performance liquid chromatography (UHPLC) coupled with a photodiode array (PDA) detector. Currently, chromatography coupled with mass spectrometry is preferred since it allows to achieve better specificity and sensitivity [11]. However, it is an expensive equipment that not all labs have. The chromatography coupled with a mass detector is a less expensive and more intuitive alternative for benefiting from the specificity of the mass [12]. Our UHPLC is equipped with this detector, but no method was described in the literature to quantify cotinine using such equipment. Therefore, the method was developed and results in comparable performances than the ones reported for mass spectrometry.

Following this first development, we aimed to use the same strategy to be able to assess more globally the individuals' tobacco status. The second developed method allowed to

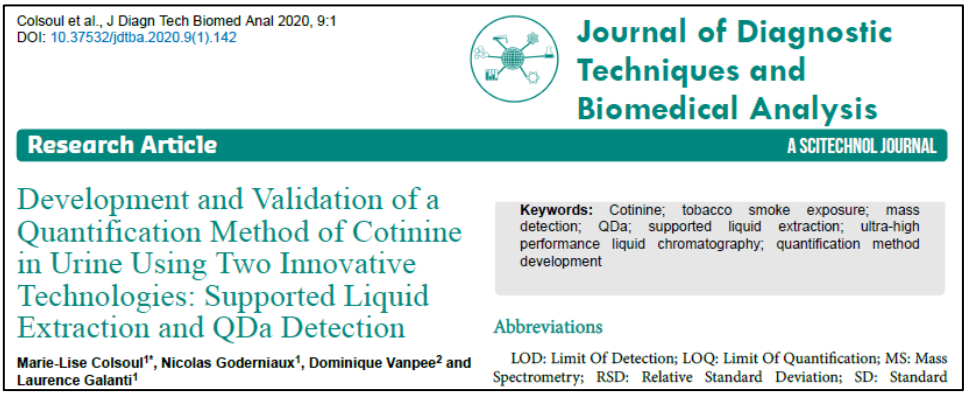
measure simultaneously five tobacco alkaloids in urine: nicotine and its metabolites – cotinine and trans-3'-hydroxycotinine (3HC) – and minor alkaloids – anatabine and anabasine. The minor alkaloids are particularly of interest since they are only present in tobacco and absent in nicotine-containing medications. Therefore, the detection of these alkaloids in participants' urine surely implied tobacco exposure [8,13]. The challenge of this development was to be able to detect the small concentrations of the minor alkaloids with our mass detector. All the reported quantification methods in the literature resort to a mass spectrometer, which is supposed to achieve the best sensitivity [14–16]. However, our new developed method allows to quantify small concentrations of the five alkaloids and, particularly, small concentrations of the minor alkaloids.

Finally, we had to adapt a commercial kit to quantify malondialdehyde (MDA), which is a biomarker of lipid peroxidation occurring in case of oxidative stress [17,18]. We performed the MDA extraction step using the kit [19], but the chromatographic separation was adapted for a UHPLC system rather than a high-performance liquid chromatography (HPLC) system. It allowed faster analysis and improved resolution. The UHPLC was connected with a fluorescence detector. The selection of the new chromatographic parameters are discussed in this chapter.

1.1 Urinary cotinine measurement

Development and validation of a quantification method of cotinine in urine using two innovative technologies: Supported Liquid Extraction and QDa detection

Paper published in Journal of Diagnostic Techniques and Biomedical Analysis, 2020, 9:1



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Journal of Diagnostic Techniques and Biomedical Analysis

Research Article

A SCITECHNOL JOURNAL

Development and Validation of a Quantification Method of Cotinine in Urine Using Two Innovative Technologies: Supported Liquid Extraction and QDa Detection

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Keywords: Cotinine; tobacco smoke exposure; mass detection; QDa; supported liquid extraction; ultra-high performance liquid chromatography; quantification method development

Abbreviations

LOD: Limit Of Detection; LOQ: Limit Of Quantification; MS: Mass Spectrometry; RSD: Relative Standard Deviation; SD: Standard

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1.1.1 Abstract

Objective: Cotinine is the best biomarker of tobacco smoke exposure because of its long half-life. Several methods are developed to quantify cotinine in biological fluids, including high performance liquid chromatography. This method requires an extraction step in order to clean-up the sample and concentrate the analyte of interest.

The aim of this study was to optimise the extraction step and to develop an easy, reproducible and specific method to measure cotinine in urine.

Methods: An extraction of neutrals was chosen and performed by Supported Liquid Extraction (SLE). SLE consists in liquid-liquid extraction in the presence of a sorbent enabling efficient extraction with less organic solvent and without any emulsion formation. Urine were basified by treatment with NH_4OH in order to neutralise cotinine before loading on SLE plate. After drying, neutrals were eluted with a mix containing dichloromethane and isopropyl alcohol. Solvent was then evaporated and samples were reconstituted with water.

Detection of cotinine was performed by mass detection using a QDa detector after UHPLC separation with a C18 column at a flow rate of 0.4 ml/min. A gradient elution of H_2O + 0.1% NH_4OH and CH_3CN was used. The method was validated based on linearity, precision, recovery and limits of detection and quantification.

Results: The range of linearity was 0.001 µg/ml - 5 µg/ml with a determination coefficient of 0.997. The precision was evaluated by the relative standard deviation (RSD) for intra- and inter-assay and was below 5% and 10% respectively. 3 levels of concentration were tested to assess the recovery rate which was consistent and higher than 96%.

Conclusion: Cotinine concentration can be measured in urine by SLE extraction and UHPLC-QDa detection. This method is easy, reproducible and allows quantification of low concentrations. It is a good solution to assess patients' tobacco smoke exposure in medical laboratory.

1.1.2 Introduction

With more than 7 million deaths per year in the world, tobacco smoking is the most preventable cause of death [20]. Some biological parameters allow to evaluate tobacco smoke exposure, especially nicotine, a specific alkaloid that is the most responsible of the tobacco physical dependence [21].

Nicotine is mainly metabolised into cotinine that is widely used as biomarker in various body fluids due to its longer half-life than the one of nicotine [22]. It allows to distinguish tobacco users from non-users [22,23]. Cotinine can be quantified by different methods, including chromatography, considered the reference method, but that requires pre-treatments such as liquid-liquid extraction, solid-phase extraction (SPE), acid precipitation, centrifugation and filtration. These methods can be time-consuming, expensive or complex [24]. Chromatography is often coupled with ultraviolet detector, single quadrupole mass spectrometry (MS) or tandem MS/MS that is more sensitive and selective [15]. However, it requires expensive laboratory instrumentation.

The aim of this study is to develop an easy, rapid and sensitive method to measure cotinine in urine by ultra-high performance liquid chromatography (UHPLC) connected with a mass detector. The mass detector is more intuitive and less expensive than a mass spectrometer while benefiting from the mass spectral information [12]. The pre-treatment used in this project to clean-up the sample is a Supported Liquid Extraction (SLE), a relatively unknown alternative approach to liquid-liquid extraction. Samples are loaded on a support (highly purified diatomaceous earth or synthetic), the aqueous phase is adsorbed onto the surface, then neutral analytes are eluted by an organic solvent. The support allows an intimate contact between the aqueous and organic phases, possibly leading to analyte recoveries higher than the ones obtained with liquid-liquid extraction [25,26].

1.1.3 Materials and methods

Solutions preparation

Cotinine solution

A 10 mg/ml solution of cotinine was prepared by diluting 19.5 mg of cotinine (Sigma-Aldrich, lot 120M4046V, Saint-Louis, USA) in 1.95 ml of water. This solution was then diluted 1:100 by making up 1 ml of solution to 100 ml with water. The final 10 µg/ml working solution was obtained after a 1:10 dilution in water.

Internal standard

A 1 g/L solution of 2-phenylimidazole was prepared by diluting 100 mg of 2-phenylimidazole (Acros Organics, lot A0283801, Molinons, France) in 100 ml of methanol (Biosolve, lot 1328761, Dieuze, France). The solution was then diluted 3:1000 in water to obtain the 3 mg/L working solution.

Calibrators and controls preparation

Five calibrators (0.05, 0.25, 1.25, 2.5 and 5 µg/ml) and two control solutions (0.25 and 2.5 µg/ml) were prepared from the 10 µg/ml working solution of cotinine by dilutions in water. A quadratic calibration curve was performed each day of analysis with a weighting factor 1/X.

Samples pre-treatment

The patients' urines were frozen immediately after collection and centrifuged at 2150 g for 5 min (Heraeus multifuge 1S, Thermo Scientific, USA) after the thawing at room temperature the day of analysis. 200 µl of urine, calibrator or control were mixed with 10 µl of 3 mg/L internal standard, 50 µl NH_4OH 1M (Sigma-Aldrich, lot BCBX5442, Saint-Louis, USA) and 40 µl of water. Samples were then thoroughly vortex-mixed.

Supported Liquid Extraction (SLE)

Pre-treated samples were loaded on a 400 µl Strata DE 96-Well plate (Phenomenex, lot M01129, Torrance, California) and a drying time of 6 minutes was waited. The elution was performed with 3 x 600 µl of dichloromethane/isopropyl alcohol 95/5 (v/v) (Biosolve, lot 10042731 and lot 1238751 respectively, Dieuze, France). The collected eluate was then evaporated to dryness at 60°C for 75 minutes (SPD121P-230, Thermo Scientific, USA) and reconstituted in 100 µl of water for 10 minutes on an orbital shaker.

Chromatographic conditions

Analyses were performed with an ultra-high performance liquid chromatography (UHPLC) system coupled with a QDa detector (Acquity, Waters, Milford, USA). Results were acquired and processed by Empower 3 (Waters). 0.3 µl of extracted samples were injected on a reverse phase column (Acquity UPLC BEH C18 130 Å 1.7 µm 2.1 mm X 100 mm, lot 0327382972, Waters) connected to a pre-column (Acquity UPLC BEH C18 VanGuard Pre-column 130 Å 1.7 µm 2.1 mm X 5 mm, lot 0311380081, Waters) and heated at 40°C. The mobile phase consisted of 0.1% NH_4OH in water (v/v) (Sigma-Aldrich, lot BCBX5442, Saint-Louis, USA) and acetonitrile (Biosolve, lot 1303351, Dieuze, France) and was used in gradient at a flow rate of 0.4 ml/min. The run started with 5% of acetonitrile for 2 minutes, acetronitrile was then gradually increased to 100% in 4 minutes. These conditions were kept for 1.5 min before reequilibrating the column for 2.5 min. The QDa detector was configured to positively ionise samples with a capillary voltage of 1.5 kV and a source temperature of 600°C. Cotinine (m/z: 177.3) and the internal standard (m/z: 145.1) were detected by single ion recording with a cone voltage of 15 V.

Method validation

Linearity

The linearity was evaluated from two-fold serial dilutions in water of a 5 µg/ml solution of cotinine. 200 µl of each dilution (n=13) underwent the extraction step and were analysed just as the samples.

Precision

The precision was evaluated by 20 measurements of two cotinine concentration levels (0.25 and 2.5 µg/ml) within-day (repeatability) and between-day (intermediate precision). These solutions corresponded to control solutions used each analysis. The precision was expressed by the relative standard deviation (RSD) between the measurements.

Accuracy

The accuracy was determined by calculating the bias between the mean of results obtained in the inter-assay experiment and the target value. It was calculated for two levels of concentration (0.25 and 2.5 µg/ml).

$$Bias\ (\%) = \frac{C_{mean} - C_{target}}{C_{target}} * 100$$

Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) were estimated from the background noise at the retention time of cotinine in 10 blank injections (purified water). LOD and LOQ were calculated as follows: LOD = mean + 3 * standard deviation (SD) and LOQ = mean + 10 * SD

Recovery

The percentage of recovery was assessed by spiking urine with small amounts of cotinine (0.2, 0.5 and 1 µg/ml). The samples were extracted and then injected in triplicate. The mean of the injections was calculated and compared to the expected value.

$$Recovery\ (\%) = 100 - \left(\frac{C_{expected} - C_{mean}}{C_{expected}}\right) * 100$$

Carry-over

Carry-over was evaluated by 3 injections of a high concentrated solution of cotinine (H) (5 µg/ml) followed by 3 injections of a low concentrated solution of cotinine (L) (0.05 µg/ml).

$$Carry - over\ (\%) = \frac{L_1 - L_3}{mean\ H - L_3} * 100$$

Robustness

Small variations in method parameters were performed in order to test the robustness of the method. The extraction and the injection of 5 calibrators, 2 controls and 3 urine samples was carried out with:

- 3 SLE eluant compositions: dichloromethane/isopropyl alcohol 95.5/4.5, 95/5 and 94.5/5.5 (v/v).
- 3 evaporation durations: 70, 75 and 80 minutes.
- 3 reconstitution durations: 5, 10 and 15 minutes.
- 3 mobile phases (variation of the aqueous phase): 0.098, 0.1 and 0.102% NH_4OH

1.1.4 Results

Chromatographic conditions determination

The examination of cotinine and 2-phenylimidazole (internal standard) structures suggested a reverse phase analysis given that molecules were both hydrophobic (log P: cotinine 0.21, 2-phenylimidazole 1.88), thereby a C18 column was chosen. Separation could be achieved according to two approaches: at acidic pH with both molecules positively charged or at alkaline pH with neutral molecules. The first approach involved an attention to the residual silanol groups (pKa: 3.5) to avoid interactions with the positive charge of cotinine and 2-phenylimidazole. A column with a polar modified particle surface (Luna Omega 1.6 µm Polar C18, Phenomenex) was tested to improve the retention of the analytes by interactions with

polar functional groups. 10 mM ammonium formate + formic acid (pH 3) was used in gradient with acetonitrile + 0.1% formic acid to elute analytes. Peaks tailing was observed, despite a low pH to avoid secondary interactions (Figure 1A). Therefore, the second approach was chosen to limit tailing and separation was performed on a BEH C18 1.7 µm column (Waters) with an alkaline mobile phase (0.1% NH_4OH in water and acetonitrile in gradient) (Figure 1B).

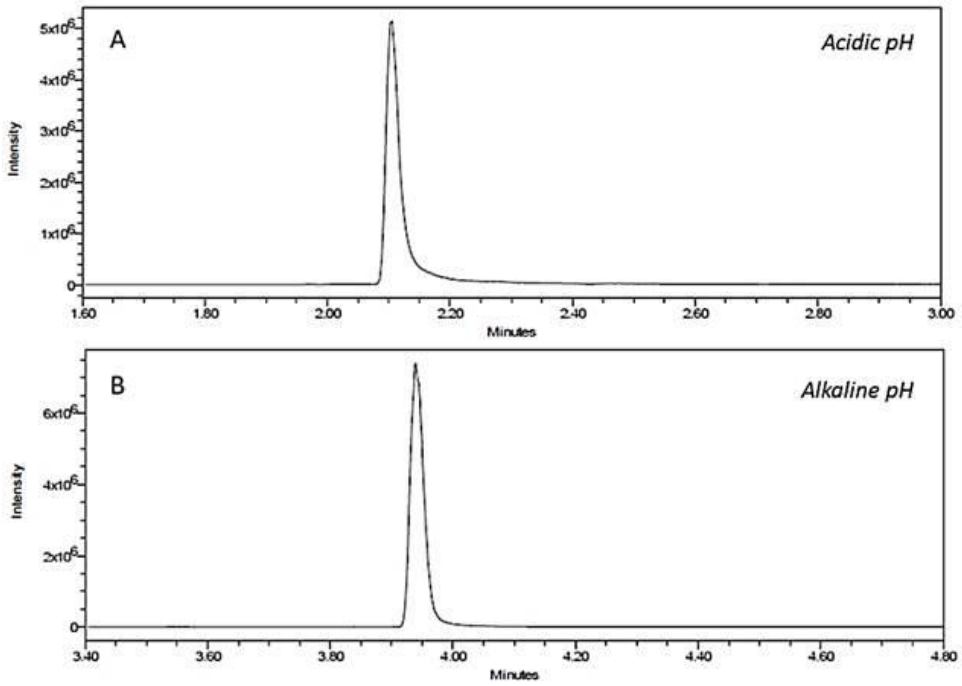


Figure 1 : Cotinine chromatogram obtained with an acidic mobile phase (A) or with an alkaline mobile phase (B)

QDa settings were optimised by varying the cone voltage (10, 15, 20 and 30 V), the positive capillary voltage (0.8, 1 and 1.5 kV) and the source temperature (400, 500 and 600°C). Parameters that gave the greatest peak area for both cotinine and internal standard were chosen: cone voltage = 15 V, capillary voltage = 1.5 kV and source temperature = 600°C.

Extraction method selection

Different extraction methods were tested: liquid-liquid extraction with chloroform, Solid Phase Extraction (SPE) (Strata-X-C, Phenomenex and Oasis MCX, Waters) and Supported Liquid Extraction (SLE) (Novum and Strata DE, Phenomenex). The liquid-liquid extraction showed a cotinine recovery varying from 70 to 88% that was not consistent for all tested concentrations (Table 1). Moreover, this method required the use of important volumes of toxic solvents and involved results variation due to the phases separation difficulty. The other methods were tested on 96 well plates allowing the easy treatment of many samples with smaller volumes of toxic solvents and without any delicate step as the phases separation. The two SPE plates tested showed poor recovery rates (Table 1), requiring a better optimisation of the extraction steps. However, SLE was prefered thanks to its easy and quick use: no conditionning, equilibrating and washing steps are required. Strata-DE plate was finally chosen and allowed a good recovery rate and the quantification of low concentrations (Table 1).

Table 1: Cotinine recovery and lower limit of the linearity range for the different tested extraction methods

	Liquid-liquid extraction	**SPE 96 Well Plate**		**SLE 96 Well Plate**	
		Strata-X-C	**Oasis MCX**	**Novum**	**Strata-DE**
Recovery (%)	70-88	18	15	66	96
Lower limit of the linearity range (µg/ml)	-	-	-	0.039	0.001

SLE optimisation

SLE does not required a lot of optimisation, only the sample pre-treatment and the elution solvent can be modified. The sample dilution before the loading on the plate was tested with water and with NH_4OH 0.5 M and cotinine recovery was 2 times bigger when diluting

with NH_4OH. The volume of diluent was chosen to comply with the maximal loading volume (300 µl). Elution was also performed with ethyl acetate before selecting dichloromethane/isopropyl alcohol 95/5 as eluent to achieve the best recovery rate.

Validation of the method

The final developed method allowed to measure cotinine corrected by an internal standard (Figure 2). A linear relationship was observed between the measured cotinine concentration and the expected cotinine concentration over a range from 0.001 µg/ml to 5 µg/ml (Figure 3). The regression equation was $y = 0.876x + 0.0416$ with $r^2=0.997$. The within-day variation was <3.3% and the between-day variation was <8.8% (Table 2). The bias between the mean of between-day results and the target value was <6.3%. LOD and LOQ were both calculated at 0.000 µg/ml based on the background noise. Therefore the LOQ was defined as the lower limit of the linearity range (0.001 µg/ml). The recovery was consistent for all concentrations tested and was >96%. No carry-over (0%) was observed for the injection of 3 low concentration samples after 3 high concentration samples. All variations tested in method parameters showed the robustness of the method given that results variation was <7.4% which is lower than the between-day variation.

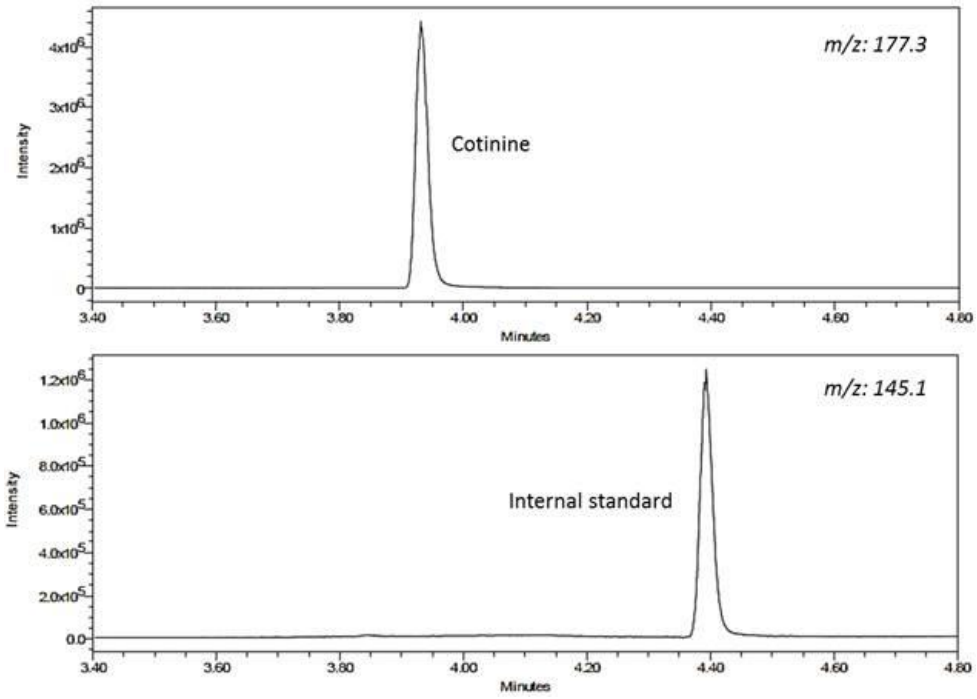


Figure 2: Chromatograms of cotinine and internal standard

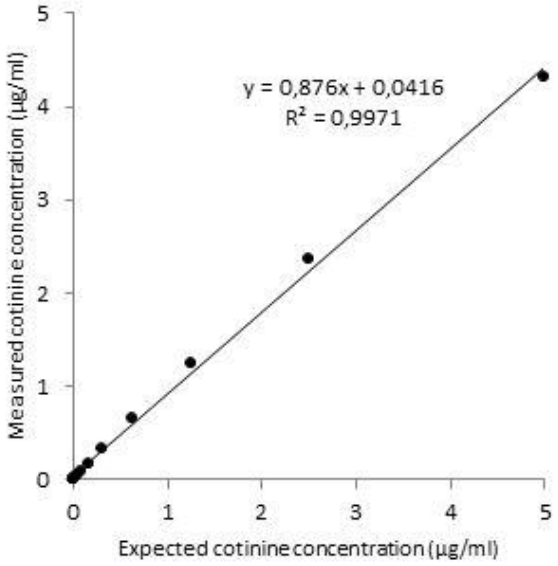


Figure 3: Linearity of the method over a range from 0.001 µg/ml to 5 µg/ml

Table 2: Within-day and between-day precision of the method

	Within-day precision (n=20)			**Between-day precision (n=20)**		
	Mean (µg/ml)	**SD (µg/ml)**	**RSD (%)**	**Mean (µg/ml)**	**SD (µg/ml)**	**RSD (%)**
Low concentration (0.25 µg/ml)	0.294	0.005	1.561	0.266	0.023	8.755
High concentration (2.5 µg/ml)	2.739	0.091	3.310	2.503	0.128	5.102

1.1.5 Discussion

The method developed in this study allows cotinine quantification in urine by a rapid and easy extraction step following by a mass detection with a QDa detector. Cotinine is a biomarker commonly used to distinguish tobacco users from non users and evaluate the extent of tobacco smoke exposure [22]. This analyte can be measured in several biological matrices (urine, blood, saliva, hair, etc.) but urine was selected in this study because its collection is non-invasive and most of all, the cotinine concentration is four to six times higher in urine than in blood or saliva. It allows to detect low concentration exposure [27].

Various techniques are showed in the literature to quantify cotinine including immunoassays, gas chromatography and high performance liquid chromatography [21,27–32]. Chromatography is usually prefered because of its good specificity. Currently, chromatography is increasingly coupled with mass spectrometry to achieve high specificity and sensitivity of analysis [11]. However, the cost of this equipment and the required training can curb laboratories. An alternative is the use of a mass detector (QDa, Waters) that is intuitive and considerably less expensive while benefiting from the specifity of the mass [12].

The quantification range obtained with this described method are satisfactory compared with the one obtained by mass spectrometry in the literature. The linear range for this method was 0.001-5 µg/ml, allowing the quantification of high concentrations as well as low concentrations as in the passive exposure case (0.005-0.02 µg/ml [33]). The linearity range obtained with HPLC-tandem mass spectrometry in urine is varying depending of the studies, for example, 0.002-6 µg/ml [11], 0.01-3 µg/ml [34] or 0.0002-4 µg/ml [16]. The other chromatographic specifications were also suitable to validate the developed method: the precision is <15%, the bias is <15%, the recovery is >70% and the carry-over is <0.05% [35,36].

Several extraction techniques were tested before choosing the SLE which consists in liquid-liquid extraction in the presence of a sorbent enabling an efficient extraction with less organic solvent and without any emulsion formation [25,26]. This method is easier and faster to implement than SPE and provided a good cotinine recovery in this study. Urine samples were basified before the loading on the SLE plate in order to neutralise cotinine that is then eluted with the organic solvent. For this reason, the recovery rate was better when pre-diluting samples with NH_4OH rather than with water.

The extraction step implies the use of an internal standard to take the cotinine loss into account. Usually, analyses by mass spectrometry resort to deuterated internal standards that have the same extraction recovery, ionisation rate and retention time as the molecule of interest [37]. In the case of cotinine extraction, cotinine-d_3 can be used as internal standard [11,16,34]. Nevertheless, the cost of this molecule is high. This study used 2-phenylimidazol as internal standard that has similarities of structure with cotinine and whose the price is approximately 3 times lower than the one of cotinine-d_3. The intermediate precision of the cotinine quantification with this developed method showed a maximal variation of 8.8% while the variation observed for the cotinine quantification by HPLC-tandem mass spectrometry is for example 3.6% [11], 4.4% [34] or 7.9% [16]. The variation obtained with this developed method is possibly lightly higher, but is anyway <15%, the maximal recommended intermediate precision in HPLC methods [35], indicating that 2-phenylimidazol is suitable as internal standard.

This study offers the prospect of developing quantification methods of other tobacco biomarkers by UHPLC analysis, such as trans-3'-hydroxycotinine, anabasine and anatabine, with mass detection after an SLE step [13,38].

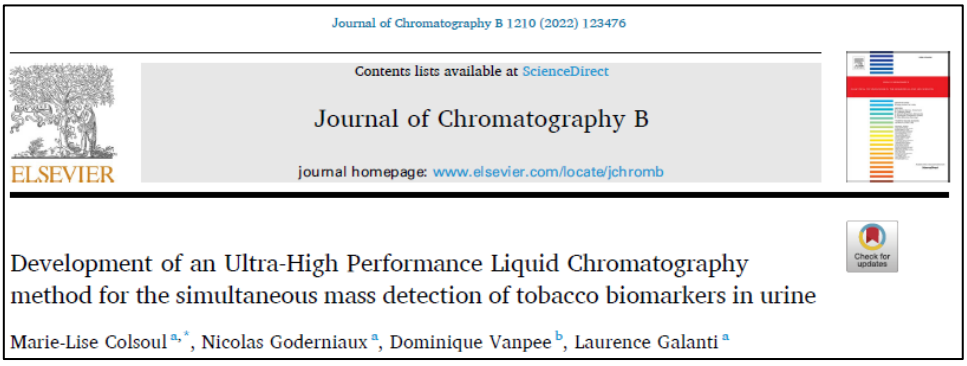
1.1.6 Conclusion

A quantification method of cotinine in urine was developed by ultra-high performance liquid chromatography coupled with a mass detector. This method is a good alternative to the detection by a mass spectrometer and allows laboratories to assess easily patients' tobacco smoke exposure without investing in expensive instrumentation. The pre-treatment was performed by Supported Liquid Extraction that is easy and quick. The method described is reproducible and allows quantification of low and high concentrations of cotinine. Therefore, it can be used both for assessing passive and active smoking.

1.2 Simultaneous detection of five tobacco biomarkers in urine

Development of an Ultra-High Performance Liquid Chromatography method for the simultaneous mass detection of tobacco biomarkers in urine

Paper published in Journal of Chromatography B, 2022, 1210:123476



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1.2.1 Abstract

The quantification of tobacco exposure biomarkers is relevant to follow the patients' tobacco use. They allow to discriminate between tobacco users, non-users, passive smokers, and nicotine products users, such as in nicotine replacement therapy. The aim of this study was to develop and validate a quantification method of tobacco biomarkers of choice – nicotine, cotinine, trans-3'-hydroxycotinine, anatabine and anabasine – in urine. The challenge was to develop an easy and rapid liquid chromatography method requiring only one extraction step and allowing simultaneous detections. Some methods are described in the literature but need specific investment in terms of instrumentation and users training. Here, the developed method had to be carried out with instrumentation easily accessible for medical laboratories. The extraction of the analytes was performed by Supported Liquid Extraction (SLE), which consists in liquid-liquid extraction but supported by a sorbent. It allows to insure efficient neutrals extraction with less organic solvent and without any emulsion formation. 200 µl of basified urine – analytes of interest are neutral in this condition – were loaded on Novum SLE 96-Well Plates (Phenomenex) and analytes were eluted with 1% formic acid in dichloromethane/propan-2-ol (95/5). After solvent evaporation, samples were reconstituted with 100 µl of water for injection. A mass detector (QDa, Waters) was used to detect analytes, this pre-optimised quadrupole mass analyser being less expensive and requiring less adjustments than traditional mass spectrometers while benefiting of the reliability of mass spectral data. This detector was integrated after an Ultra-high performance liquid chromatography (UHPLC) separation on a BEH C18 column (Waters) at a flow rate of 0.5 ml/min. A gradient elution of H_2O (pH 10 with NH_4OH) and CH_3CN was used. Finally, the developed method was validated. This new method is conclusive to assess the patients' tobacco exposure and is easy to implement in medical laboratories.

1.2.2 Introduction

Tobacco consumption is associated with many adverse effects on health. Studies have shown a relationship between the tobacco smoke exposure and the risk of developing smoking-related diseases. Therefore, accurate measure of the active or passive tobacco smoke exposure is essential to evaluate these risks linked to the individual impregnation with compounds of tobacco. It is also important to consider in case of organ transplant, elective surgery, insurance purposes or smoking cessation programs to optimise treatments or assess the compliance to the nicotine replacement therapy (NRT). The most reliable method of evaluation consists in the quantification of specific biomarkers [9,13,39].

Nicotine is the major alkaloid in tobacco and is responsible for tobacco addiction. Nicotine can be measured in body fluids to relate exposure to nicotine by smoked tobacco, smokeless tobacco, or delivery of non-tobacco nicotine, including NRT and alternative nicotine products [40–42]. However, nicotine is more representative of recent than long-term exposure given its short half-life – approximately 2-3 hours in the blood [9,10,14]. Cotinine, the main metabolite of nicotine, is the biomarker of choice for nicotine uptake thanks to its longer half-life – 15-19 hours in different body fluids. It allows to distinguish between users and non-users of nicotine but also to evaluate passive exposure [9,10,27]. Cotinine is metabolised by CYP2A6 to trans-3'-hydroxycotinine. The CYP2A6 activity can be reflected by the trans-3'-hydroxycotinine to cotinine ratio called "the nicotine metabolite ratio" (NMR) which correlates highly with nicotine clearance. Several studies have reported the relationship between the NMR and the response to treatments for smoking cessation. It appears that treatments could be personalised depending on the nicotine metabolism rate [43–45]. Although excellent biomarkers for tobacco use, all these major alkaloids do not allow to discriminate tobacco users and people using non-tobacco nicotine delivery products, such as electronic nicotine delivery devices (ENDS), gums, transdermal patches, nasal/oral sprays, inhalers, etc. [8,10,13,40]. Anatabine and anabasine are two minor alkaloids not derived metabolically from nicotine. They are present in tobacco but absent in nicotine-containing medications. Therefore, they can validate tobacco abstinence in NRT users [10,13,46].

Different analytical methods have been developed to quantify tobacco biomarkers, including colorimetry, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA) and chromatography. The latter is generally prefered given its high sensitivity and specificity and the possibility of quantifying several biomarkers simultaneously [9,47,48]. Chromatography is often coupled with mass spectrometry to detect low alkaloids concentrations, especially for anatabine and anabasine presents in low concentrations in body fluids [14,23,24]. However, this instrumentation is expensive and requires specific training for users, which can make this technique difficult to implement in medical laboratories.

The aim of this study is to develop an easy and sensitive method to measure nicotine, its metabolites – cotinine and trans-3'-hydroxycotinine – and the minor alkaloids – anatabine and anabasine – in urine. Although the specificity of these biomarkers and their low concentrations in body fluids, an easily accessible instrumentation for routine medical laboratories is used: an ultra-high performance liquid chromatography (UHPLC) system connected with a mass detector. This detector generates quality mass spectral data, but is as intuitive as an optical detector and less expensive than a mass spectrometer [12]. Samples are cleaned up by a Supported Liquid Extraction (SLE) prior to the chromatography analysis. This is an alternative to liquid-liquid extraction where samples are loaded onto a support which retains the entire sample before the elution of neutral analytes with an immiscible organic solvent. It allows to treat easily many samples simultaneously and to achieve high analytes recoveries [26,49].

1.2.3 Materials and Methods

Chemicals and reagents

Nicotine, cotinine, nicotine-d_4 and cotinine-d_3 were obtained from Merck (Darmstadt, Germany), trans-3'-hydroxycotinine, trans-3'-hydroxycotinine-d_3, anabasine-d_4 and anatabine-d_4 from Toronto Research Chemicals (North York, Canada), and anabasine and anatabine from Cayman Chemical (Ann Arbor, Michigan, USA).

Ammonium hydroxide solution (28% NH_3 in H_2O) were purchased from Merck. Acetonitrile, dichloromethane, propan-2-ol, methanol, water, and formic acid of ULC/MS grade were provided by Biosolve (Dieuze, France).

Internal standard and stock solutions

Stock solutions were prepared by dilutions in water: 100 µg/ml nicotine, 100 µg/ml cotinine, 1 mg/ml trans-3'-hydroxycotinine, 2 µg/ml anatabine, and 2 µg/ml anabasine. Internal standard solutions were also prepared by dilutions in water: 35 µg/ml nicotine-d_4, 35 µg/ml cotinine-d_3, 140 µg/ml trans-3'-hydroxycotinine-d_3, 210 ng/ml anatabine-d4, and 210 ng/ml anabasine-d_4.

Calibrators and controls preparation

Six calibrators and three controls were prepared each day of analysis from dilutions of the stock solutions in water (Table 1). Calibrators allowed the establishment of a quadratic calibration curve with a weighting factor 1/X.

Table 1: Calibrators and controls concentrations

	Nicotine (µg/ml)	**Cotinine (µg/ml)**	**Trans-3'-hydroxycotinine (µg/ml)**	**Anatabine (ng/ml)**	**Anabasine (ng/ml)**
Calibrator 1	0.02	0.002	0.02	0.029	0.029
Calibrator 2	0.05	0.05	0.2	0.3	0.3
Calibrator 3	0.25	0.25	1	1.5	1.5
Calibrator 4	1.25	1.25	5	7.5	7.5
Calibrator 5	2.5	2.5	10	15	15
Calibrator 6	5	5	20	30	30
Control 1	0.05	0.005	0.05	0.075	0.075
Control 2	0.25	0.25	1	1.5	1.5
Control 3	2.5	2.5	10	15	15

Samples pre-treatment

Urine samples were frozen at -20°C immediately after collection. The day of analysis, they were thawed at room temperature and then centrifuged at 2150 g for 5 minutes (Heraeus multifuge 1S, Thermo Scientific, USA). 200 µl were mixed with 10 µl of each internal standard solutions and 100 µl of 8% ammonium hydroxide solution (NH_4OH), prepared from 28% NH_4OH and water. Calibrators and controls were treated in the same way as the samples.

Supported Liquid Extraction (SLE)

Pre-treated samples were loaded onto a Novum SLE MAX 96-Well Plate (Phenomenex, Torrance, California) in order to extract neutrals, including the 5 analytes of interest with their deuterated internal standard. A vacuum was applied until the samples had completely entered the sorbent. A drying time of 5 min was waited before the elution with 3 x 600 µl of 1% formic acid in dichloromethane/propan-2-ol 95/5 (v/v). A vacuum of approximately 10 seconds ensured the complete extraction. The eluate was evaporated to dryness at 45°C for 80 min (SPD121P-230, Thermo Scientific, USA) and reconstituted in 100 µl of water for 10 min on an orbital shaker.

Chromatographic conditions

The chromatographic system was composed of a H-Class UPLC connected to a QDa detector (Acquity, Waters, Milford, USA). Data acquisition and processing were performed by Empower 3 (Waters). The reversed-phase column (Acquity UPLC BEH C18 130 Å 1.7 µm 2.1 mm X 100 mm, Waters) and the pre-column (Acquity UPLC BEH C18 VanGuard Pre-column 130 Å 1.7 µm 2.1 mm X 5 mm, Waters) were held at 30°C for the analysis. Analytes were eluted by the mobile phase consisting in a gradient of NH_4OH in water (pH 10) and acetonitrile at a flow rate of 0.5 ml/min. The run time was 6 min. Initial proportion of acetonitrile was 10% and reached 30% by a gradual increase during 1.5 min. The conditions were kept for 1.5 min before an isocratic plateau of 1.5 min at 90%. The column was then reequilibrated for 1.5 min before the next injection. The needle wash solvent was a mixture

of 0.1% formic acid in water/acetonitrile/methanol/propan-2-ol (1/1/1/1). The probe temperature and the capillary voltage of the detector was set at 600°C and 0.5 kV. The cone voltage was 15 V for all molecules. Analytes were quantified using single ion recording at m/z 163 (nicotine), 167 (nicotine-d_4), 177 (cotinine), 180 (cotinine-d_3), 193 (trans-3'-hydroxycotinine), 196 (trans-3'-hydroxycotinine-d_3), 161 (anatabine), 165 (anatabine-d_4), 163 (anabasine), and 167 (anabasine-d_4). Extracted samples were injected 2 times with 2 different injection volumes: 0.3 µl to quantify major alkaloids (nicotine, cotinine, and trans-3'-hydroxycotinine) and 10 µl to quantify minor alkaloids (anatabine and anabasine).

Method validation

The method was validated according to ISO15189 standard and the Guideline on bioanalytical method validation of European Medicines Agency (EMA) [50]. Samples containing all analytes were used in order to take influence caused by other analytes into account. Depending on the experiment and its objective, samples consisted in urine samples or mixtures of all analytes in water. These latters allowed to define precisely initial concentrations in tobacco biomarkers.

Linearity

Two-fold serial dilutions in water of a high concentrated solution (10 µg/ml nicotine, 10 µg/ml cotinine, 40 µg/ml trans-3'-hydroxycotinine, 30 ng/ml anabasine, and 30 ng/ml anatabine) were performed to determine the method linearity range. Each dilution (n=15) was extracted in the same way as samples before being injected in the UPLC system.

Lower limit of quantification

The lower limit of quantification (LLOQ) of each analyte was determined in a non-smoker's urine sample. The sample was fortified with small amounts of analytes. Three runs of 5 repetitions were performed to evaluate precision and accuracy of LLOQ.

Precision

The precision was evaluated at two levels (intra- and inter-assay) for three solutions corresponding to control samples (Table 1). Three runs of 5 repetitions were performed for control 1. For controls 2 and 3, a single run of twenty repetitions (intra-assay) and twenty runs (inter-assay) were performed. The Relative Standard Deviation (RSD) was calculated to determine the imprecision.

Accuracy

The accuracy was estimated by the bias between the mean of inter-assay results and the target value. It was calculated for three concentrations, corresponding to control samples.

$$Bias\ (\%) = \frac{C_{mean} - C_{target}}{C_{target}} * 100$$

Recovery

Three urine samples were extracted and injected to determine their initial tobacco components concentrations before being spiked with increasing amounts of analytes (Table 2). Measured concentrations were compared to expected concentrations to determine the recovery rate of the method.

$$Recovery\ (\%) = 100 - \left(\frac{C_{expected} - C_{measured}}{C_{expected}}\right) * 100$$

Table 2: Concentrations added to three urine samples to determine the recovery rate of the method

	Nicotine (µg/ml)	**Cotinine (µg/ml)**	**Trans-3'-hydroxycotinine (µg/ml)**	**Anatabine (ng/ml)**	**Anabasine (ng/ml)**
Initial	0	0	0	0	0
Spike 1	0.2	0.2	0.8	1.2	1.2
Spike 2	0.5	0.5	2	3	3
Spike 3	1	1	4	6	6

Matrix effect

The matrix effect was evaluated by comparing the slopes of each calibration curve obtained for standard solutions (see "Linearity") and the ones obtained when spiking matrix with different concentrations of analytes (see "Recovery") [51].

Carry-over

The carry-over was assessed by the quantification of 3 low concentrated solutions (L), corresponding to calibrator 1, after the injection of 3 high concentrated solutions (H), corresponding to calibrator 6.

$$Carry - over\ (\%) = \frac{L_1 - L_3}{H_{mean} - L_3} * 100$$

Robustness

Small deliberate variations in method parameters were performed to test the robustness of the method. The RSD was calculated for the quantification of tobacco components in three urine samples treated with variations of:

- Evaporation durations: 75, 80 and 85 min
- SLE eluant compositions: 1% formic acid in dichloromethane/propan-2-ol 95.5/4.5, 95/5 and 94.5/5.5 (v/v)
- pH of the aqueous phase for the elution gradient: 9.8, 10 and 10.2

1.2.4 Results

Chromatographic conditions determination

The general hydrophobic nature of the analytes of interest were indicated by the logarithm of the partition coefficient (log P: nicotine 1.16, cotinine 0.21, trans-3'-hydroxycotinine -0.73, anatabine 1.02, and anabasine 1.22) that were predominantly positive. Therefore, a reversed-phase column was selected (C18). The aqueous phase using for the elution gradient was adjusted to pH 10 regarding the microspecies distributions of all analytes. This pH was suitable to separate each analyte as only one neutral species, therefore avoiding tailing and secondary interactions. Two aqueous phase were tested: water and 8 mM ammonium bicarbonate, both adjusted to pH 10 with NH_4OH. It appeared that peaks intensity was higher when using water (Figure 1A). The retention time was stable even without the use of a buffer (RSD = 0.1%). Peaks intensity was also higher when using acetonitrile as organic phase compared to methanol (Figure 1B).

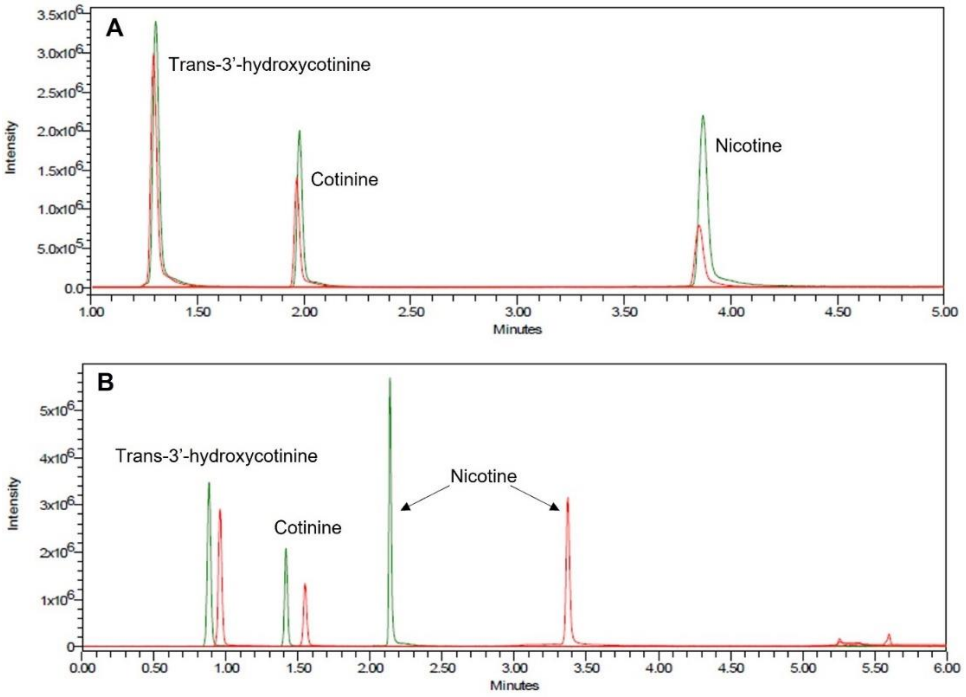

Figure 1: Chromatograms of trans-3'-hydroxycotinine, cotinine and nicotine. Anatabine and anabasine are not showed due to their low intensity compared to major alkaloids. A: Chromatograms obtained with water pH 10 (green) or ammonium bicarbonate pH 10 (red) as aqueous phase and methanol as organic phase. B: Chromatograms obtained with water pH 10 as aqueous phase and acetonitrile (green) or methanol (red) as organic phase.

The elution gradient was optimised to achieve a resolution higher than 1.5 (2.12) between anabasine and nicotine, observed on the same mass channel given their identical mass. To that end, the elution gradient was composed of an isocratic plateau between 1.5 and 3 minutes at 30% acetonitrile. Different mass detector parameters were tested in order to select the ones giving the highest peaks intensity: capillary voltage = 0.5kV, source temperature = 600°C and cone voltage = 15V.

Extraction method selection and optimisation

A previous study about the urinary cotinine quantification suggested the use of SLE to clean-up samples before chromatographic analysis [52]. This technique was slightly modified: Novum SLE MAX 96-Well Plate was used instead of Strata DE 96-Well plate (Phenomenex) and 1% of formic acid was added in the elution solvent (dichloromethane/propan-2-ol 95/5). SLE has proven to be suitable for the simultaneous extraction of the 5 target analytes given that good recovery rates and low limits of quantification could be achieved (see "Method validation"). The analytes were loaded onto the sorbent in their non-ionised form by basifying urine samples with a solution of ammonia. It allowed to recover analytes in the organic elution solvent. The elution was performed in 3 times to improve the recovery.

Internal standards selection

A single internal standard – 2-phenylimidazole – was first used to correct for variability due to analytes loss during the extraction. However, the extraction recovery was not reproducible between samples (nicotine: 70-116%, cotinine: 80-103%, trans-3'-hydroxycotinine: 95-100%, anatabine: 82-110%, and anabasine: 89-103%). These results indicated that 2-phenylimidazole was not suitable as internal standard due to a matrix effect not well compensated. Deuterated internal standards were then selected and allowed to achieve reproducible and high recovery rates (see "Method validation"). Concentrations of internal standards added to samples were calculated to reach 1/3 of the peak area of analytes in the most concentrated calibrator (calibrator 6).

Method validation

Representative chromatograms obtained with the developed method are shown in Figure 2. The method was validated according to linearity, lower limit of quantification (LLOQ), precision, accuracy, recovery, matrix effect, carry-over, and robustness. The linearity range obtained for each analyte is showed in Table 3. LLOQ were 0.02 µg/ml (nicotine and trans-

3'-hydroxycotinine), 0.002 µg/ml (cotinine), and 0.029 ng/ml (anatabine and anabasine) with maximal bias and RSD of 15.2% (intra- and inter-assay).

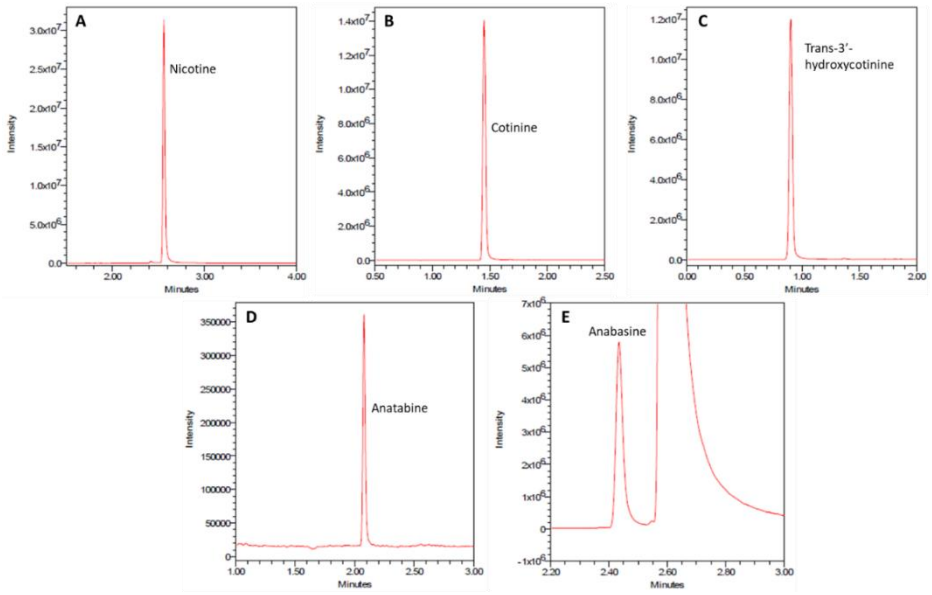

Figure 2: Representative chromatograms of 2.5 µg/ml nicotine (A), 2.5 µg/ml cotinine (B), 10 µg/ml trans-3'-hydroxycotinine (C), 15 ng/ml anatabine (D), and 15 ng/ml anabasine (E) in water.

Table 3: Linearity range for each analyte with the equation and the determination coefficient of the linear relationship between the measured and the expected concentrations.

Analyte	**Linearity**		
	Range	**Equation**	**R^2**
Nicotine (µg/ml)	0.02-10	y=0.9059x-0.023	0.9999
Cotinine (µg/ml)	0.002-10	y=1.0419x+0.0116	0.9991
Trans-3'-hydroxycotinine (µg/ml)	0.02-40	y=0.9114x+0.0591	0.9996
Anatabine (ng/ml)	0.029-30	y=1.0284x+0.1121	0.9974
Anabasine (ng/ml)	0.029-30	y=1.0888x-0.0371	1

The precision was evaluated at three concentration levels by the RSD that was <9.8% (intra- and inter-assay) for all analytes. The maximal bias, representative of the accuracy, was 13.6%. The recovery rates were high and reproducible (Recovery rates mean (RSD): nicotine 104% (4.0%), cotinine 104% (1.4%), trans-3'-hydroxycotinine 102% (2.9%), anatabine 100% (5.1%), and anabasine 95% (7.9%)). The slopes of spiking curves were 1.0231 for nicotine, 1.0340 for cotinine, 1.2616 for trans-3'-hydroxycotinine, 1.0033 for anatabine, and 0.9577 for anabasine. When compared to the slopes obtained for standard solutions in the linearity experiment (Table 3), slight ion enhancement was observed for the quantification of nicotine and trans-3'-hydroxycotinine while ion-suppression was observed for the quantification of anabasine. However, these matrix effects were low and well compensated given that all slopes were almost similar and close to 1. The carry-over was <0.04% for each analyte thanks to the use of a suitable needle wash solvent. The small variations of the method parameters showed the robustness of the method. The analytes concentrations quantified with different evaporation times showed a RSD of 4.1%. The variation of the SLE eluent composition led to a RSD of 2.0%. Finally, the different pH of the aqueous phase led to a RSD of 3.0%. These RSD are below the one obtained in the inter-assay experiment.

1.2.5 Discussion

This study describes a new method to quantify tobacco biomarkers allowing the distinction between tobacco and non-tobacco-derived nicotine users. The major limitation for using existing methods reported in the literature for medical laboratories is the significant investment needed to implement the technique. Actually, tobacco exposure biomarkers are frequently quantified by chromatography coupled with mass spectrometry [14,24,31]. The method here described resorts to chromatography connected with a mass detector, which is less expensive and easier to use while benefiting of the good specificity of the mass information [12].

The extraction technique is also simplified compared to liquid-liquid extraction (LLE) or Solid-Phase Extraction (SPE) commonly proposed [23,24]. The extraction step is here performed by SLE which is easier and more reproducible than LLE due to the use of a sorbent

avoiding emulsion formation and manual separation of the aqueous and organic phases. Another advantage is less consumption of toxic organic solvent [25,26,49]. SPE is more selective through anion or cation exchange moieties linked to the sorbent but implies more steps – conditionning, several washes, and elution – which all require optimisation. Therefore, it is time consuming for both method development and routine use [53]. Results obtained showed that SLE was suitable as clean-up method since it allows the simultaneous extraction of the five analytes of interest with an insignificant matrix effect and high and reproducible recovery rates. The recommendation of recovery rates higher than 70% with a maximal RSD of 15% is respected [35].

The analytes quantification ranges obtained with this method seem to allow the differenciation between urine samples of active tobacco (combustible or noncombustible) users, passive smokers, non-tobacco-derived nicotine users, and non-users. Cut-off limits to distinguish these populations are not well established but some are proposed in the literature [54]. Active tobacco users seem to have urinary concentrations >0.1 µg/ml for nicotine, >0.073 µg/ml for cotinine, >0.4 µg/ml for trans-3'-hydroxycotinine, and >2ng/ml for anatabine and anabasine. Recent researches based on data from Population Assessment of Tobacco and Health (PATH) Study reported that dual users generally had higher nicotine exposure than exclusive cigarettes or ENDS users [42]. Concentrations found in NRT users are similar to active users, but anatabine and anabasine concentrations are <2ng/ml [13,34]. Therefore, anatabine and anabasine levels can be used to validate tobacco abstinence during NRT [13]. It should be noted that it is possible to find measurable amounts of these minor alkaloids in urine of commercial nicotine products users. Nicotine contained in these products is less purified than the pharmaceutical grade nicotine and is almost always derived from tobacco. Only some ENDS fluids contain synthetic nicotine and, therefore, do not contain any anatabine and anabasine [8]. Passive smokers present concentrations <0.02 µg/ml for nicotine [34], <0.02 µg/ml [34] or <0.05 µg/ml [54] for cotinine depending on the authors and <0.05 µg/ml for trans-3'-hydroxycotinine [34]. Finally, urinary concentrations for non-users with no passive exposure would be <0.002 µg/ml for nicotine, <0.005 µg/ml for cotinine, <0.05 µg/ml for trans-3'-hydroxycotinine, and

<2 ng/ml for anatabine and anabasine [13,33,34]. Even though the lower quantification limit of nicotine with this new method is 0.02 µg/ml and consequently does not allow the quantification of typical values of passive exposure, cotinine and trans-3'-hydroxycotinine allow to characterise this type of exposure and to distinguish between tobacco non-users with or without passive exposure.

The developed method could be validated according to ISO15189 standard and the Guideline on bioanalytical method validation of EMA [50]. Linearity ranges were obtained from results for which back-calculated concentrations were within 15% of the nominal value. The precision was expressed as the "imprecision" which was <15% for the three studied concentrations. It was important to evaluate it at different concentrations since this parameter is concentration-dependent. The maximal bias observed was <15% of the nominal value, meaning a suitable accuracy. The precision and the bias were <20% for LLOQ. Carry-over was <0.05%. The RSD obtained in the robustness experiments were lower than the one of the inter-assay experiment, indicating that these deliberate variations had no significant effect on the analytes quantification [35,36,55].

A first limitation of this new method is its inability to distinguish cigarette smokers from users of other tobacco forms, such as cigars, pipes, water pipes, etc. (combustible tobacco) but also chewing tobacco, heated tobacco, dry snuff, etc. (non-combustible tobacco). Biomarkers other than tobacco alkaloids should be used for this goal. Exhaled carbon monoxide (CO), volatile organic chemicals (VOCs), and polycyclic aromatic hydrocarbons (PAHs) are proposed to relate the use of combustible tobacco, but they are not specific to tobacco smoking [8,10]. A second limitation is that the tobacco biomarkers analysed in this method development do not allow the identification of the NRT form used. Among nicotine users, the method can discriminate between NRT products users and alternative nicotine products, such as ENDS, nicotine gel, etc. since nicotine in commercial products is less purified and thus contains measurable concentrations of anabasine and anatabine. However, the distinction between vaping and NRT can be not clear given that some ENDS fluids contains synthetic nicotine and therefore, do not contain any anabasine and anatabine. Currently, no biomarkers specific for ENDS use are available [8,40]. To evaluate

patients' tobacco status and the source of tobacco or nicotine used, it is therefore essential to analyse a combination of several biomarkers more or less specific to tobacco. This method of quantification of nicotine, cotinine, trans-3'-hydroxycotinine, anatabine, and anabasine, suitable for medical laboratory use could help in this purpose. The suitability of the method to distinguish tobacco users from non-tobacco users should be tested in real conditions using urine samples from different tobacco-use groups. A clinical study is in progress to that end.

1.2.6 Conclusion

A novel quantification method of tobacco biomarkers allowing the differentiation of tobacco-derived nicotine users, non-tobacco-derived nicotine users, passive smokers, and non-users was developed and validated in this study. This method is rapid and easily accessible for medical laboratories through the use of SLE and liquid chromatography coupled with a mass detector. The method can be used for assessing tobacco exposure and the related health risks but also in the context of smoking cessation programs. The tobacco biomarkers quantification is of interest to adjust treatments depending on the objectified individual impregnation or to validate tobacco abstinence during NRT.

1.3 Quantitative analysis of malondialdehyde in plasma

1.3.1 Introduction

"Oxidative stress" is defined as the imbalance between the generation of reactive oxygen species (ROS) and the cell antioxidant capacity in favor of prooxidants [17,56]. ROS, produced by enzymatic and non-enzymatic systems from molecular oxygen (O_2), are highly reactive towards several classes of biomolecules. Damages to DNA, proteins, carbohydrates, and lipids may occur [18,19]. Particularly, the reaction of ROS with lipids such as polyunsaturated fatty acids (PUFAs) is called "lipid peroxidation". It results in lipid peroxyl radicals that decompose into various reaction products, such as 4-hydroxy-2-nonenal (HNE), F2-isoprostanes and malondialdehyde (MDA) (Figure 1) [17,18]. In this way, MDA is widely used as a biomarker of lipid peroxidation [17].

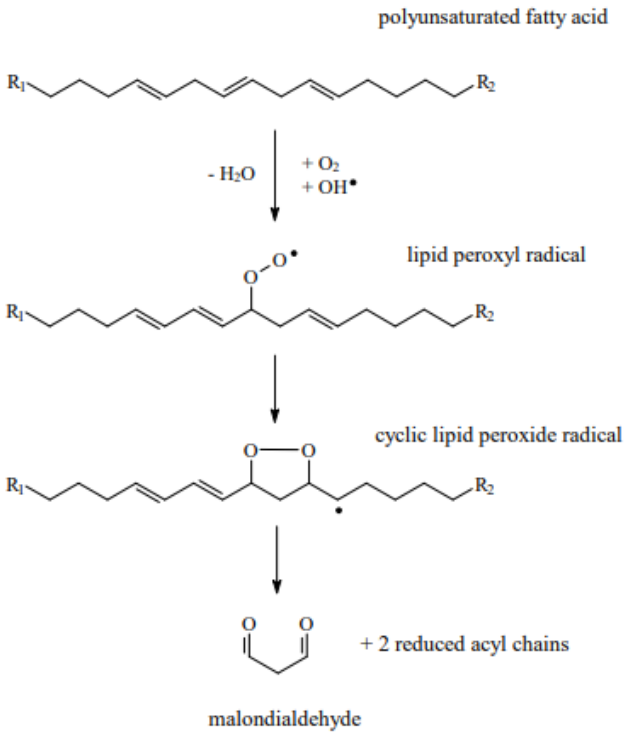

Figure 1: Formation of malondialdehyde by lipid peroxidation [19]

A technical kit from Chromsystems (Munich, Germany) allows to measure MDA in plasma by a high-performance liquid chromatography (HPLC) method and a fluorescence detection after a derivatisation. The objective of this development was to adapt the commercial kit to an UHPLC separation to improve resolution and enable faster analysis.

1.3.2 Materials and Methods

Calibrators and controls preparation

The lyophilised plasma calibrator (reference number 67003, Chromsystems) and the two lyophilised plasma controls (0095 and 0096, Chromsystems) were reconstituted with 0.5 ml purified water. They were gently shaken at room temperature for about 5-10 minutes before being treated as samples according to the preparation procedure. Their MDA concentrations depended on the batch. Typical concentrations were 29 µg/l for calibrator, 17 µg/l for control 1 and 56 µg/l for control 2. The system was calibrated each day of analysis and validated by the injection of the two controls.

Samples preparation procedure

Plasma samples were frozen at -20°C prior to batch analyses. All reagents were provided in the commercial kit (67000, Chromsystems). One hundred µl plasma were mixed with 500 µl precipitation reagent into a light protected reaction vial and vortex mixed for 10 s. It was centrifuged for 5 min at 16 000 g (Biofuge Pico, Heraeus, Germany). Five hundred µl of supernatant were transferred into a derivatisation vial. One hundred µl derivatisation reagent were added before mixing and incubation for 60 min at 95°C. Vials were cooled down and 500 µl neutralisation buffer were added. After mixing, 5 µl were injected into the UHPLC system.

Chromatographic conditions

The chromatographic system was an H-Class UPLC coupled with a fluorescence detector (Acquity, Waters, Milford, USA). Data were acquired and processed by Empower 3 (Waters). The separation was achieved on a reverse phase column (Luna Omega 1.6 µm C18 100Å

100X2.1 mm, Phenomenex, Torrance, California) maintained at 30°C. The mobile phase was a mixture of solvents from Chromsystems (67001) used at a flow rate of 0.3 ml/min. The run time was 4 min. The needle wash solvent consisted in a mixture of water and methanol (50/50 v/v). Detection was performed with excitation wavelength of 515 nm and emission wavelength of 553 nm. Due to the lack of internal standard, samples were injected in duplicate and relative standard deviation (RSD) between the two injections was checked.

Method validation

The adapted method was validated according to ISO15189 standard. Linearity, precision, limits of detection and quantification, accuracy, recovery, and carry-over were evaluated.

Linearity

Two-fold serial dilutions (n=10) in water of a high concentrated solution (112 µg/l) were performed. Each dilution was treated according to the samples preparation procedure before being injected in the UHPLC system.

Precision

Intra- and inter-assay precisions were determined on two levels of concentration, corresponding to control samples. A single run of twenty repetitions (intra-assay) and fourteen runs (inter-assay) were performed. The imprecision was expressed through the RSD.

Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) were estimated from the background noise at the retention time of MDA in 10 blank injections (mobile phase). They were calculated as follows: LOD=mean+3*standard deviation (SD) and LOQ=mean+10*SD.

Accuracy

The accuracy was estimated at two concentration levels, corresponding to control samples, by the bias between the mean of inter-assay results and the target value.

$$Bias\ (\%) = \frac{C_{mean} - C_{target}}{C_{target}} * 100$$

Recovery

The MDA concentration in a plasma sample was measured before its fortification by increasing amounts of MDA (2, 5, and 10 µg/l). Solutions for fortification were prepared from the calibrator sample of Chromsystems. All samples (initial and spiked) were analysed in triplicate. Means of measured values were compared to expected concentrations to determine the recovery rate of the method.

$$Recovery\ (\%) = 100 - \left(\frac{C_{expected} - C_{measured}}{C_{expected}}\right) * 100$$

Carry-over

The carry-over was assessed by three measurements of a high (H) concentrated samples (corresponding to control 2, theoretical concentration 56 µg/l) followed by three measurements of a blank (B) (mobile phase).

$$Carry - over\ (\%) = \frac{B_1 - B_3}{H_{mean} - B_3} * 100$$

1.3.3 Results and discussion

The analysis of MDA after derivatisation could be achieved on a classical C18 column that was suitable for an UHPLC system. A representative chromatogram is shown in Figure 2.

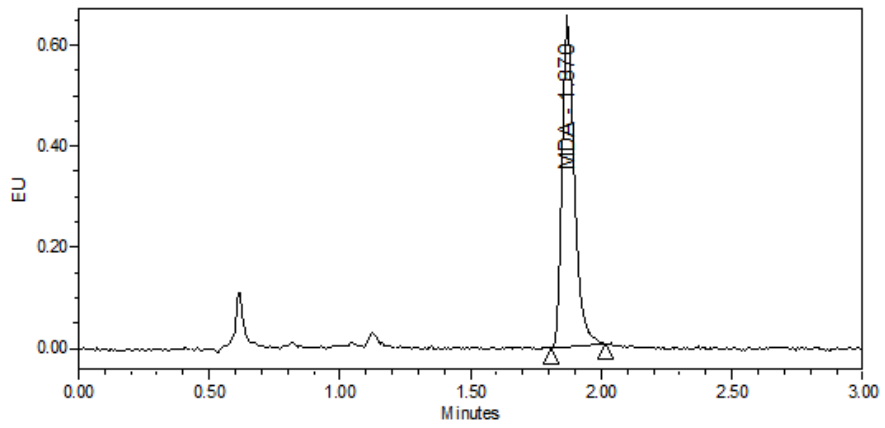

Figure 2: Representative chromatogram of 29.1 µg/ml malondialdehyde in a plasma sample

A linear relation between the peak area and the concentration was observed over the range 3.5 – 110 µg/l. The regression equation was y=1.03x+1.49 with r^2=0.9998. The measured values did not exceed 9% of the nominal value. The accuracy criterion, reported by Rosing et al. (2000), of maximal deviation of 15% for a chromatographic method is fulfilled [35]. This range allowed the quantification of concentrations considered normal (<36 µg/l [19]) or pathological (>36 µg/l [19]). The imprecision was evaluated at two concentration levels by the RSD that was <1.6% and <8.9% for intra- and inter-assay respectively. It fulfilled the criterion RSD <15% [35,36]. The limits of detection and quantification calculated based on the background noise were 0.118 µg/l and 0.320 µg/l respectively. Given that the lower limit of the linearity range was higher, the limit of quantification of this method was fixed at 3.5 µg/l. The bias, representing the accuracy, was <9% and thereby respected the criterion <15% [35,36]. The recovery was >96% for each tested level of concentration and the RSD between the replicates was <11%. This experiment was validated because the recovery was higher than 70% with a maximal variation of 15% [35]. No carry-over (0%) was observed.

1.3.4 Conclusion

The adapted method allows the quantification of MDA in plasma on an UHPLC system connected to a fluorescence detector. It allows to reflect the extent of lipid peroxidation occuring in case of oxidative stress. The extraction and the derivatisation of MDA was performed using the commercial kit from Chromsystems. The chromatographic analysis was then achieved with a classical UHPLC C18 column in a run time of 4 minutes. The method was validated according to ISO15189 standard.

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Chapter II. Comparision smokers *versus* non-smokers: assessment of the endothelial dysfunction and discrimination biomarkers

Although the implied mechanisms remain not clear, the link between smoking and cardiovascular diseases (CVDs) is conclusive [1]. It is related to endothelial dysfunction, which is a pathological condition associated with an imbalance of oxidising and inflammatory molecules [2].

An objective method to characterise smoking-induced endothelial dysfunction is the comparison of specific biomarkers in smokers and non-smokers [2,3]. Some of them are already shown affected by tobacco smoking, but other ones remain not studied [2]. In this project, we selected various biomarkers reflecting endothelial activation, oxidative stress, and inflammation. Lipid profile was also studied given that it is known to be altered when smoking [4]. All parameters were compared in smokers and non-smokers. Results showed that endothelial activation, inflammation, and oxidative stress were increased in smokers. Lipid profile was also found impacted in smokers. It confirmed the processes reported in the literature through biomarkers not yet studied.

Collected data were then used to evaluate the possibility of discriminating between smokers and non-smokers using endothelial dysfunction-related biomarkers. Usually, it is performed with tobacco exposure biomarkers and the most commonly used is cotinine [5]. But, the emergence of non-tobacco nicotine delivery products, such as electronic nicotine delivery devices (ENDS), involves the need of identification of new non-nicotinic biomarkers reflecting tobacco use [6]. Anatabine and anabasine, two minor tobacco alkaloids, were suggested to this end [7]. However, several studies have put in doubt the utility of these biomarkers due to their low clinical sensitivity [8,9]. In this chapter, we show the discrimination features of tobacco biomarkers, including anatabine and anabasine, and we compare them to the ones of endothelial dysfunction-related biomarkers. We found that the best sensitivity and specificity were achieved with tobacco biomarkers. We determined new cutoff values for anatabine and anabasine and these adapted values allowed us to achieve a sensitivity as high as the one associated with nicotine-derived biomarkers. We

showed in this way the interest of these two biomarkers. However, none of the studied biomarkers of endothelial function alone allowed both good sensitivity and specificity in discriminating smokers from non-smokers.

In order to improve the discrimination based on biomarkers other than tobacco, we tested several combinations of them. Two models could be identified and allowed >84% of correct classifications. These results offer the prospect of determining individuals' smoking status by classic parameters of blood test, without the need of specific equipment for quantifying tobacco biomarkers.

2.1 Study of endothelial dysfunction-related biomarkers in smokers and non-smokers

Biological effect of cigarette smoking in endothelial dysfunction: study of biomarkers of endothelial function, oxidative stress, inflammation and lipids

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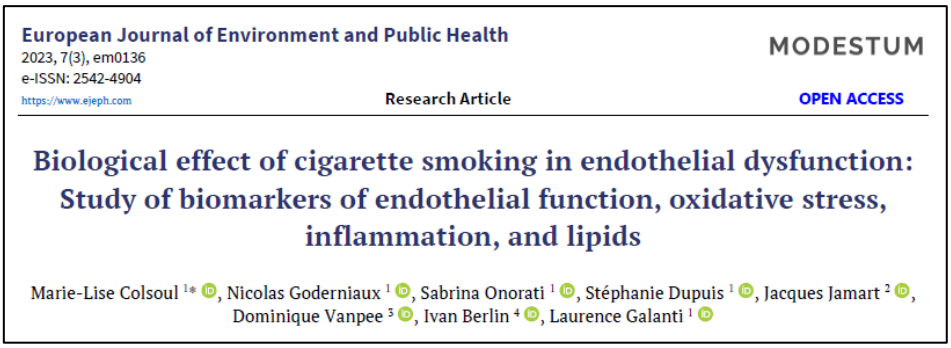

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Biological effect of cigarette smoking in endothelial dysfunction: Study of biomarkers of endothelial function, oxidative stress, inflammation, and lipids

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2.1.1 Abstract

Aims: Tobacco use is involved in endothelial dysfunction, a key marker of cardiovascular diseases. The contribution of tobacco use in their development is assessed by endothelial dysfunction-related biomarkers in smokers and non-smokers.

Methods: 138 smokers and 83 non-smokers were recruited. Parameters reflecting the endothelial function, lipid profile and oxidative and inflammatory status, were quantified. Data were used to determine their ability to differentiate smokers and non-smokers.

Results: Elevation of inflammation and oxidative stress as well as alteration of endothelial function and lipids profile in smokers were observed. Two biomarkers combinations, including one implying only routine parameters, were identified and allowed to correctly classify >84% of cases.

Conclusions: Oxidative status, inflammatory status, and lipids profile were shown altered in smokers, leading to endothelial dysfunction. Endothelial dysfunction-related biomarkers were assessed in terms of their ability to discriminate smokers from non-smokers. The possibility of discrimination based only on classic parameters of blood test appeared conceivable.

2.1.2 Introduction

Tobacco use kills more than 8 million people each year worldwide and remains the most preventable cause of death. It is associated with different pathological conditions and is, especially, the major preventable risk factor for the development and progression of cardiovascular diseases (CVDs), which are the main cause of death globally [10,11]. Tobacco consumption is responsible for oxidative stress, inflammation, lipid profile and haemodynamic alterations, and hypercoagulability, all being involved in endothelial dysfunction, the first step of CVDs development [1,2,12].

The effect of tobacco on endothelial dysfunction can be assessed by several techniques more or less invasive: intra-arterial/venous infusion of vasoactive substances, flow

mediated dilation (FMD), endothelial cell culture, etc. [3]. Another possibility is the evaluation of different biomarkers related to the endothelial function and associated processes such as oxidative stress, inflammation, and lipid profile modifications [1–3].

A multitude of biomarkers can be used to reflect the endothelial function, oxidative status, inflammatory status, and lipids [3,13–15]. Among them, some are commonly used to assess endothelial dysfunction and are already shown altered by tobacco smoking. Different studies have reported an increased level of inflammatory biomarkers, including C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor alpha (TNF-α) in smokers [2,16,17]. On the other hand, level of IL-10, an anti-inflammatory cytokine, is shown lower in smokers [2,17]. This elevation of inflammation is associated with the increase of leukocyte-endothelial cell interactions mediated by the soluble vascular cell adhesion molecule (sVCAM) and the soluble intercellular adhesion molecule (sICAM). Both are shown at higher concentration in smokers than in non-smokers [16]. Although the involved mechanisms remain not clear, tobacco smoking seems to lead to dyslipidemia: researches have indicated that smokers have higher cholesterol, triglycerides, and low-density lipoprotein (LDL) levels as well as lower high-density lipoprotein (HDL) level [16]. Oxidative modification, including oxidation of LDL, is also increased in smokers. Products of lipid peroxidation, such as malondialdehyde (MDA), and anti-oxidized LDL (oxLDL) antibodies are reported at higher concentrations in smokers [16,18]. Oxidative stress in smokers is also associated with weaker antioxidant defences: vitamins C and E levels are found lower than in non-smokers [2,18].

Although some parameters have been addressed, the influence of smoking on other ones remains unknown. The aim of this study is to propose a more extensive study of endothelial dysfunction-related biomarkers in smokers versus non-smokers. Oxidative stress is evaluated through the quantification of some antioxidants (vitamins A/E/C, β-carotene, coenzyme Q10, uric acid), pro-oxidants or indicators of oxidation (iron, MDA, anti-oxLDL antibodies), and regulatory enzymes (ferritin, transferrin, glutathione peroxidase (GPX), superoxide dismutase (SOD)). Inflammatory status is estimated by the concentration measurement of high-sensitivity CRP (hsCRP), monocyte chemoattractant protein 1

(MCP-1), IL 6/8/10, and TNF-α. Asymmetric dimethylarginine (ADMA) – interferent in the production of the vasodilator nitric oxide (NO), sVCAM, and sICAM levels are quantified to reflect the endothelial function. Lipid profile is monitored by the concentration quantification of cholesterol, HDL, LDL, triglycerides, lipoprotein (a) (Lp(a)), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), and small dense LDL cholesterol (sdLDL). For the first time to our knowledge, these biomarkers are also addressed in terms of their ability in discriminating between smokers and non-smokers. Their discrimination features are compared with the ones of five tobacco exposure biomarkers: nicotine and its metabolites – cotinine and trans-3'-hydroxycotinine (3HC) – anatabine and anabasine. Finally, the combination of several biological markers – other than tobacco – to distinguish the two groups is evaluated.

2.1.3 Materials and Methods

Participants

As shown in Table 1, one hundred and thirty-eight active cigarette smokers, 64 men and 74 women between 26 to 80 years of age, were recruited at the tobaccology unit of the Centre Hospitalier Universitaire of Catholic University of Louvain (Namur, Belgium). Ten of them suffered from chronic obstructive pulmonary disease (COPD) without acute pathology. The average consumption was 19 cigarettes per day (range: 3-60) and the time since last cigarette varied from 0.5 to 2 hours. Eighty-three healthy long-term non-smokers, 40 men and 43 women between 21 to 83 years of age, were recruited on a voluntary basis through advertisement in the same hospital. The potential presence of clinical pathology that may influence the endothelial function was addressed based on participants' health database and the analysis of confounding factors for the two groups. Informed consent was provided from all participants. The project was approved by the hospital ethics committee and was led according to the Declaration of Helsinki and the Good Clinical Practice guidelines [19].

Table 1: Baseline characteristics

	Smokers (n=138)	**Non-smokers** (n=83)
Age, years	55 (44 – 64)	55 (38 – 64)
Male, n (%)	64 (46.38)	40 (48.19)
BMI (kg/m²)	25.0 (21.2 – 28.8)	26.5 (24.6 – 28.9)
COPD, n (%)	10 (7.25)	0 (0)

Data are expressed as median (Interquartile range) or n (%). BMI, body mass index; COPD, chronic obstructive pulmonary disease.

Clinical Protocol

Blood samples and urine from smokers and non-smokers were collected to measure biological parameters of interest. Several aliquots (urine, heparin whole blood, and EDTA whole blood) were taken and immediately frozen at -20°C for later batch analyses. Blood tubes were then centrifuged (15 minutes, 5±3°C, 5950 g, SL16R, Thermo Scientific, USA) to collect serum, sodium fluoride plasma and heparinised plasma. Analyses were performed on this fresh material or on serum/plasma aliquots stored at -20°C for chromatographic assays and enzyme linked immunosorbent assays (ELISA).

Instruments for quantitative analyses

VITROS® 5600 System and VITROS® 3600 System from Ortho Clinical Diagnostics (Raritan, USA) were used for measurement of some parameters directly after blood collection and centrifugation.

The chromatographic assays were performed using a high-performance liquid chromatography (HPLC) system coupled with an ultraviolet (UV) detector (Alliance 2695, detector 2489, Waters, Milford, USA) and an ultra-high performance liquid chromatography (UHPLC) system (Acquity UPLC® H-Class), coupled with a photodiode array detector (Acquity PDA), fluorescence detector (Acquity FLR), or a mass detector (Acquity QDa). Acquisition

and process software was Empower 3 (Waters). Runs were carried out in batch and systems were calibrated each day of analysis.

ELISA assays were also performed in batch and required a microplate strip washer (ELx50, Agilent, Santa Clara, USA) for washing steps and an absorbance microplate reader (800TS, Agilent) for readings.

Other batch analyses were performed on BN ProSpec® System (Siemens Healthcare, Saint-Denis Cedex, France).

Biological parameters evaluation

Smoking status

The exhaled carbon monoxide (CO) amount was measured for smokers by electrochemistry with a Smokerlyzer® monitor (Bedfont Scientific Ltd, Harrietsham, UK). Five tobacco biomarkers (nicotine, cotinine, 3HC, anatabine, and anabasine) were quantified in urine by a previously described method (UHPLC-mass detection) [20]. These parameters were quantified for smokers but also for non-smokers to validate the absence of tobacco biomarkers.

Oxidative stress

Serum iron and uric acid concentrations were assessed by colorimetry on VITROS 5600. Serum ferritin was measured by immunoassay on VITROS 3600 and serum transferrin was quantified on VITROS 5600 by turbidimetry (reagent pack TFTUR-C00, DiAgam, Ghislenghien, Belgium).

GPX and SOD were both analysed on VITROS 5600 in batch, through an enzymatic method (kit RANSEL, Randox, Crumlin, UK) for GPX in heparin whole blood and through a colorimetric activity kit (kit RANSOD, Randox, Crumlin, UK) for SOD in EDTA whole blood.

HPLC connected with an UV detector allowed to detect vitamin C in plasma, coenzyme Q10 and β-carotene in serum. Respective chromsystems kits (Munich, Germany) were used.

Chromsystems kits allowed also the extraction of vitamin A, vitamin E, and malondialdehyde from plasma. Detection was performed with an UHPLC system, using an UHPLC reverse phase column (Acquity UPLC HSS T3 1.8 µm 2.1 X 50 mm, Waters) with a flow rate of 0.6 ml/min and photodiode array detection (scan 210-400 nm) for vitamins and an UHPLC reverse phase column (Luna Omega 1,6 µm C18 100Å 100X2,1 mm, Phenomenex, Torrance, USA) with a flow rate of 0.3 ml/min and fluorescence detection (excitation wavelength: 515 nm; emission wavelength: 553 nm) for malondialdehyde.

Anti-oxLDL antibodies in serum were assessed by ELISA kit (Biomedica Medizinprodukte GmbH, Wien, Austria).

Inflammatory status

MCP-1, IL-6, 8, and 10 were measured in serum through ELISA kits from Abcam B.V. (Amsterdam, Netherlands). TNF-α was assessed in serum by an ELISA kit from Tecan (IBL International GmbH, Hamburg, Germany). High-sensitivity (hs) CRP was quantified in serum by nephelometry using the BN ProSpec.

Endothelial function

ADMA, sVCAM, and sICAM were measured in serum by ELISA kits (MT-Diagnostics B.V., Etten-Leur, Netherlands).

Lipid profile

Cholesterol, HDL, LDL, and triglyceride were assessed in serum by enzymatic methods on VITROS 5600. Lp(a), ApoA1, ApoB were quantified by the BN ProSpec by nephelometry. sdLDL was measured with an ELISA kit (Cubasio, Houston, USA).

Confounding factors

Glycaemia, glutamic-pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), γ-glutamyl transferase (GGT), creatinine, albumin, and magnesium tests were performed on VITROS 5600. Matrix was fresh serum for all tests, except sodium fluoride plasma for

glycaemia. Glycohemoglobin (HbA1c) was quantified on fresh EDTA whole blood by an automated analyser based on HPLC (ADAMS A1c HA-8180V, Arkray, Amstelveen, Netherlands).

Statistical analyses

Normality of the data was rejected by Shapiro-Wilk test. Therefore, non-parametric tests were used and results were expressed by median with interquartile range into bracket. The biomarkers comparison in smokers versus non-smokers was performed by Wilcoxon rank sum test. Differences were considered significant when $P<0.05$. Logistic regressions were carried out with backward selection of variables. The diagnostic value of significant variables was assessed by sensitivity (Se), specificity (Sp), positive (PPV) and negative predictive values (NPV), Youden index [21], and area under (AUC) the receiver operating characteristic (ROC) curves. PPV and NPV were calculated based on a prevalence of smoking of 20% [22]. All analyses were performed using MedCalc® (version 20.109, MedCalc Software Ltd, Ostend, Belgium).

2.1.4 Results

Evaluation of the smoking status

The smoking status of enrolled participants was self-reported and confirmed by the CO measurement and the quantification of five tobacco biomarkers. Median concentrations are shown in Table 2. As expected, all biomarkers were significantly higher in smokers when compared with non-smokers.

Table 2: Median concentrations of tobacco biomarkers in smokers versus non-smokers

	Smokers Median (Interquartile range) (n=116)	**Non-smokers** Median (Interquartile range) (n=47)	**P**
CO (ppm)	18 (13 – 23)*	-	-
Cotinine (µg/ml)	1.21 (0.85 – 1.71)*	0.01 (0.01 – 0.01)	<0.0001
Nicotine (µg/ml)	0.581 (0.227 – 1.114)	0.055 (0.055 – 0.055)	<0.0001
3HC (µg/ml)	3.72 (1.58 – 6.07)	0.02 (0.02 – 0.02)	<0.0001
Anatabine (ng/ml)	5.595 (2.013 – 11.286)	0.029 (0.029 – 0.029)	<0.0001
Anabasine (ng/ml)	1.785 (0.559 – 7.440)	0.029 (0.029 – 0.137)	<0.0001

CO, carbon monoxide; 3HC, *trans*-3'-hydroxycotinine. *n=137

Confounding factors

Parameters that may indicate clinical pathology (diabetes, liver disease, renal failure) were studied. Although levels of GPT, creatinine, and magnesium were significantly lower in smokers than in non-smokers, there was no biological difference between the two groups (Table 3). No other statistical differences were observed in confounding factors.

Table 3: Median concentrations of confounding factors in smokers versus non-smokers

	Smokers Median (Interquartile range) (n=138)	**Non-smokers** Median (Interquartile range) (n=83)	**P**
Glycaemia (mg/dl)	92 (85 – 100)	93 (86 – 101)	0.8500
GPT (U/l)	30 (21 – 38)	35 (27 – 46)	0.0030
GOT (U/l)	25 (21 – 30)	26 (22 – 31)	0.1477
GGT (U/l)	30 (19 – 50)	25 (19 – 37)	0.0915
Creatinine (mg/dl)	0.73 (0.66 – 0.87)	0.83 (0.72 – 0.96)	0.0002
Albumin (g/l)	42.3 (40.6 – 44.6)	41.7 (39.2 – 45.0)	0.1780
Mg (mmol/l)	0.83 (0.77 – 0.88)	0.85 (0.80 – 0.89)	0.0121
HbA1c (%)	5.4 (5.2 – 5.7)	5.4 (5.1 – 5.6)	0.0913

GPT, glutamic-pyruvic transaminase; GOT, glutamic oxaloacetic transaminase; GGT, γ-glutamyl transferase; Mg, magnesium; HbA1c, glycohaemoglobin.

Evaluation of the effect of cigarette smoking on biomarkers involved in the endothelial function

Inflammatory biomarkers

Concentrations of inflammatory biomarkers in smokers and non-smokers are given in Table 4a. Increased inflammation was observed in smokers by a significant elevation in the chemokine IL-8 and hsCRP levels when compared with non-smokers. An opposite result was found for IL-10 level: an increase was observed in smokers while it is an anti-inflammatory cytokine. The medians of TNF-α concentrations in smokers and non-smokers were found identical, despite a light but significant shift in both values distributions. There were no differences for other studied biomarkers.

Endothelial biomarkers

The cell adhesion molecules, sVCAM and sICAM, were both at increased concentration in smokers (almost significant for sVCAM) compared with non-smokers (Table 4b). ADMA, interfering with the production of NO, was not found to be affected by the smoking status.

Oxidative stress biomarkers

The lipid peroxidation biomarker, MDA, was at significantly higher concentration in smokers versus non-smokers (Table 4c). Levels of uric acid, vitamin C and β-carotene, all antioxidants, showed a decrease in smokers versus non-smokers. Levels of ferritin and transferrin, both involved in the regulation of the iron level, were respectively increased and decreased in smokers. Anti-oxLDL antibodies concentration was lower in smokers. No other significant differences were found in the studied biomarkers.

Lipid profile

No differences were observed when comparing the lipid profile in smokers or in non-smokers, except the triglyceride level that was increased in smokers (Table 4d). sdLDL level showed a significant decrease in smokers.

Table 4a: Median concentrations of inflammatory biomarkers in smokers versus non-smokers

	Smokers Median (Interquartile range) (n=138)	**Non-smokers** Median (Interquartile range) (n=83)	**P**
IL-6 (pg/ml)	3.5 (2.0 – 5.3)	3.1 (1.8 – 5.8)	0.4134
IL-8 (pg/ml)	9.8 (6.2 – 15.4)	6.1 (3.2 – 10.3)	<0.0001
IL-10 (pg/ml)	0.8 (0.4 – 2.2)	0.5 (0.4 – 1.3)	0.0317
TNF-α (pg/ml)	0.15 (0.15 – 0.35)	0.15 (0.15 – 0.15)	0.0055
MCP-1 (pg/ml)	76.0 (55.4 – 127.8)	84.9 (60.0 – 106.2)	0.7315
hsCRP (mg/l)	1.6 (0.8 – 3.4)	1.2 (0.6 – 2.1)	0.0078

IL, interleukine; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; hsCRP, high-sensitivity C-reactive protein

Table 4b: Median concentrations of biomarkers of endothelial function in smokers versus non-smokers

	Smokers Median (Interquartile range) (n=138)	**Non-smokers** Median (Interquartile range) (n=80)	**P**
ADMA (µmol/l)	0.6 (0.5 – 0.8)	0.6 (0.5 – 0.7)	0.5654
sVCAM (ng/ml)	815.5 (695.6 – 1026.0)	783.7 (624.6 – 956.0)	0.0603
sICAM (ng/ml)	384.6 (302.8 – 504.8)	328.1 (254.3 – 405.1)	0.0003

ADMA, asymmetric dimethylarginine; sVCAM, soluble vascular cell adhesion molecule; sICAM, soluble intercellular adhesion molecule

Table 4c: Median concentrations of biomarkers of oxidative status in smokers versus non-smokers

	Smokers Median (Interquartile range) (n=138)	**Non-smokers** Median (Interquartile range) (n=83)	**P**
SOD (U/ml)	242.0 (213.9 – 282.0)	238.0 (191.5 – 279.5)	0.3075
GPX (U/l)	12206 (9876 – 14814)*	13166 (9875 – 14814)**	0.9464
MDA (µg/l)	7.4 (5.7 – 11.3)*	5.7 (4.9 – 6.5)**	<0.0001
Uric acid (mg/dl)	4.8 (3.8 – 5.9)	5.4 (4.3 – 6.3)	0.0032
Iron (µg/dl)	98 (73 – 121)	92 (78 – 123)	0.7519
Ferritin (ng/ml)	72.4 (37.6 – 117.0)	48.9 (20.8 – 113.5)	0.0242
Transferrin (g/l)	2.56 (2.29 – 2.81)*	2.64 (2.43 – 17.7)**	0.0094
Vitamin A (mg/l)	0.55 (0.43 – 0.69)	0.57 (0.48 – 0.71)	0.1661
Vitamin E (mg/l)	13.4 (11.1 – 16.6)	14.5 (11.6 – 17.0)	0.4425
Vitamin C (mg/l)	8.8 (4.9 – 11.7)	11.0 (8.7 – 13.9)	<0.0001
Coenzyme Q10 (µg/l)	794 (607 – 1033)	742 (582 – 952)	0.1766
β-carotene (ng/ml)	157 (104 – 247)	379 (217 – 542)	<0.0001
Anti-oxLDL (U/l)	155.6 (80.2 – 339.5)	328.5 (143.5 – 939.5)	0.0001

SOD, superoxide dismutase; GPX, glutathione peroxidase; MDA, malondialdehyde; anti-oxLDL, anti-oxidized LDL antibodies. *n=70; **n=41

Table 4d: Median concentrations of lipids in smokers versus non-smokers

	Smokers Median (Interquartile range) (n=138)	**Non-smokers** Median (Interquartile range) (n=83)	**P**
Cholesterol (mg/dl)	192 (167 – 224)	197 (170 – 227)	0.4795
HDL (mg/dl)	56 (43 – 67)	54 (48 – 68)	0.7893
LDL (mg/dl)	119 (87 – 151)	120 (97 – 155)	0.4005
Triglycerides (mg/dl)	121 (93 – 169)	104 (82 – 162)	0.0117
Lp(a) (g/l)	0.09 (0.04 – 0.31)	0.08 (0.03 – 0.25)	0.3335
ApoA1 (g/l)	1.54 (1.38 – 1.80)	1.59 (1.42 – 1.79)	0.6273
ApoB (g/l)	0.95 (0.77 – 1.15)	0.93 (0.80 – 1.14)	0.7776
sdLDL (nmol/ml)	1464 (706 – 3143)	2355 (1456 – 3294)	0.0400

HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lp(a), lipoprotein (a); Apo, apolipoprotein; sdLDL, small dense LDL

Discrimination between smokers and non-smokers based on biomarkers of tobacco, inflammation, endothelial function, oxidative stress, and lipids

Biomarkers significantly affected by the smoking status ($P<0.05$ in Table 4a,b,c,d) were evaluated in terms of their ability to differentiate smokers and non-smokers. Features of each biomarker are given in Table 5a,b,c,d,e. All tobacco exposure biomarkers showed the best features of discrimination with excellent sensitivity (>96%, except for nicotine and anabasine >87%), specificity (>97%), positive predictive value (>91%), negative predictive value (>97%), and area under the ROC curve (>0.940). The ability of discriminating was weaker for other biomarkers, however all presented negative predictive values >83%. IL-8, ferritin and transferrin allowed good sensitivity (>81%) while MDA and vitamin C allowed good specificity (>80%).

Table 5a: Features of tobacco exposure biomarkers for discriminating between smokers and non-smokers

	Se (%)	**Sp (%)**	**PPV (%)**	**NPV (%)**	**AUC**	**Cut-off**	**YI (%)**	**P**
Cotinine	100.0	100.0	100	100	1.000	>0.047 µg/ml	100.0	<0.0001
Nicotine	87.9	100.0	100	97.07	0.940	>0.055 µg/ml	87.9	<0.0001
3HC	99.1	100.0	100	99.79	0.995	>0.094 µg/ml	99.1	<0.0001
Anatabine	96.6	97.9	91.89	99.13	0.980	>0.097 ng/ml	94.4	<0.0001
Anabasine	88.8	97.9	91.24	97.22	0.954	>0.236 ng/ml	86.7	<0.0001

Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the receiver operating characteristic (ROC) curve; YI, Youden index; 3HC, trans-3'-hydroxycotinine.

Table 5b: Features of inflammatory biomarkers for discriminating between smokers and non-smokers

	Se (%)	**Sp (%)**	**PPV (%)**	**NPV (%)**	**AUC**	**Cut-off**	**YI (%)**	**P**
IL-8	81.9	46.9	27.83	91.19	0.674	>5.43 pg/ml	28.8	<0.0001
IL-10	39.1	78.8	31.52	83.81	0.585	>1.39 pg/ml	17.9	0.0317
TNF-α	47.1	77.1	33.97	85.36	0.597	>0.15 pg/ml	24.2	0.0055
hsCRP	40.6	75.9	29.62	83.63	0.607	>2.15 mg/l	16.5	0.0078

Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the receiver operating characteristic (ROC) curve; YI, Youden index; IL, interleukine; TNF, tumor necrosis factor; hsCRP, high-sensitivity C-reactive protein.

Table 5c: Features of biomarkers of endothelial function for discriminating between smokers and non-smokers

	Se (%)	**Sp (%)**	**PPV (%)**	**NPV (%)**	**AUC**	**Cut-off**	**YI (%)**	**P**
sVCAM	47.8	71.6	29.63	84.59	0.576	>856.6 ng/ml	19.4	0.0603
sICAM	62.3	65.0	30.80	87.34	0.647	>351 ng/ml	27.3	0.0003

Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the receiver operating characteristic (ROC) curve; YI, Youden index; sVCAM, soluble vascular cell adhesion molecule; sICAM, soluble intercellular adhesion molecule.

Table 5d: Features of biomarkers of oxidative status for discriminating between smokers and non-smokers

	Se (%)	**Sp (%)**	**PPV (%)**	**NPV (%)**	**AUC**	**Cut-off**	**YI (%)**	**P**
MDA	57.9	85.4	49.82	89.03	0.730	>6.7 µg/l	43.3	<0.0001
Uric acid	61.6	59.0	27.32	86.01	0.619	<5.19 mg/dl	20.6	0.0032
Ferritin	85.5	33.7	24.39	90.30	0.591	>27.1 ng/ml	19.2	0.0242
Transfer-rin	94.9	39.6	28.20	96.90	0.638	<3 g/l	34.5	0.0094
Vitamin C	48.2	80.7	38.44	86.16	0.670	<8.3 mg/l	28.9	<0.0001
β-carotene	79.7	69.9	39.82	93.23	0.769	<277.5 ng/ml	49.6	<0.0001
Anti-oxLDL	71.0	59.3	30.35	83.10	0.663	<268.1 U/l	30.3	<0.0001

Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the receiver operating characteristic (ROC) curve; YI, Youden index; MDA, malondialdehyde; anti-oxLDL, anti-oxidized LDL antibodies.

Table 5e: Features of lipid parameters for discriminating between smokers and non-smokers

	Se (%)	**Sp (%)**	**PPV (%)**	**NPV (%)**	**AUC**	**Cut-off**	**YI (%)**	**P**
Trigly-cerides	64.5	57.8	27.66	86.69	0.601	>108 mg/dl	22.3	0.0117
sdLDL	48.1	79.3	36.68	85.93	0.597	<1327 nmol/ml	27.3	0.0400

Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the receiver operating characteristic (ROC) curve; YI, Youden index; sdLDL, small dense low-density lipoprotein.

None of the studied biomarkers, other than tobacco biomarkers, showed both good sensitivity and specificity. Therefore, logistic regressions were performed to find a combination of biomarkers, including confounding factors, allowing better discrimination between smokers and non-smokers. Preliminary regressions were performed in each category of parameters. Significant variables in these selections were regrouped into a subsequent analysis to obtain the final model (Table 6, model 1). It was associated with a good area under the ROC curve (AUC=0.877) and 88.5% of cases correctly classified. Another model was carried out according to the same principle, but based only on parameters – routine parameters – easily and commonly measurable in medical laboratories (Table 6, model 2). This one was also associated with a good area under the ROC curve (AUC=0.928) and 84.3% of cases correctly classified.

Table 6: Final models obtain by logistic regressions based on all studied parameters (model 1) or on routine parameters (model 2) to discriminate between smokers and non-smokers

Model 1			**Model 2**		
Variable	**Coefficient**	**P**	**Variable**	**Coefficient**	**P**
Vitamin C	-0.237	0.0197	hsCRP	0.594	0.0058
ApoB	3.844	0.0269	Uric acid	-0.658	0.0036
HbA1c	2.470	0.0360	Transferrin	-0.774	0.0092
GPT	-0.099	0.0101	HDL	-0.048	0.0131
GGT	0.076	0.0159	Glycaemia	-0.041	0.0109
Constant	-12.320	0.0613	GOT	-0.168	0.0009
			GGT	0.036	0.0404
			Albumin	0.494	0.0001
			Constant	-5.120	0.2762

Apo, apolipoprotein; HbA1c, glycated hemoglobin; GPT, glutamic-pyruvic transaminase; GGT, gamma-glutamyltransferase; hsCRP, high-sensitivity C-reactive protein; HDL, high-density lipoprotein; GOT, glutamic oxaloacetic transaminase; GGT, γ-glutamyl transferase

2.1.5 Discussion

This research proposes an extensive study of endothelial dysfunction-related biomarkers depending on the smoking status. It is known that tobacco affects the endothelial function through its effect in associated processes, such as oxidative stress, inflammation, and changes in lipid profile, but implied mechanisms are still not clear. Since endothelial dysfunction is considered the early step in the development of CVDs, the main cause of death worldwide, it is crucial to assess the effect of tobacco in this process [1,11,23].

The self-reported participants' smoking status was objectified by the measurement of five urinary tobacco biomarkers – nicotine, cotinine, 3HC, anatabine and anabasine. Some cutoff values are proposed in the literature to differentiate smokers from non-smokers with these biomarkers: >0.1 µg/ml for nicotine, >0.073 µg/ml for cotinine, >0.4 µg/ml for 3HC, and >2

ng/ml for anatabine and anabasine [24]. Non-smoking was proven for the non-smokers group given that concentrations were lower than these cutoff values.

Cigarette smoke has proinflammatory effects by inducing the production of proinflammatory cytokines, such as TNF-α, IL-6, and IL-8 [25]. It is associated with increased circulating levels of CRP, an acute phase protein [26]. An elevation in IL-8 and hsCRP levels were indeed observed in smokers, but no difference was observed for TNF-α and IL-6 concentrations. It could be due to low sensitivity of assay kits, especially for TNF-α: 53% of recruited smokers and 77% of non-smokers had concentrations under the limit of quantification (LOQ) of 0.15 pg/ml. However, TNF-α is a primary proinflammatory cytokine that induces the production of chemokines, including IL-8, and the generation of IL-6, a secondary proinflammatory cytokine, which subsequently induces the production of acute phase proteins by the liver, such as CRP [27]. Consequently, the effects of TNF-α and IL-6 are visible through the increase of IL-8 and hsCRP levels. On the other hand, an unexpected result was obtained for IL-10, an anti-inflammatory cytokine which prevents the expression of inflammatory mediators [28]: the level was higher in smokers than in non-smokers while the opposite was shown in the study of Ugur et al. (2018) [17]. The medians difference between the two groups (0.8 for smokers versus 0.5 pg/ml for non-smokers) was, however, quite slight and probably not significant biologically. Moreover, the accurate value was not available for many participants due to the LOQ of 0.39 pg/ml (33% in the smokers' group and 43% in the non-smokers' group below the LOQ). However, another hypothesis could explain the observed results: an upregulation of the anti-inflammatory cytokine IL-10 could occur in response to the increased inflammation observed in smokers.

In endothelial dysfunction, inflammation is associated with an increased ability of endothelial cells to adhere to immunity cells, such as monocytes, macrophages, T lymphocytes, and platelets [16,29]. It leads to higher concentrations of adhesion molecules in plasma, as in smokers' plasma [2,16]. This study confirms this by the elevation of sICAM and sVCAM (almost significant) concentrations in smokers.

The proinflammatory status is also associated with oxidative stress [1,2]. It was demonstrated that cigarette smoke induces oxidative stress due to increased reactive oxygen species (ROS) production and antioxidants defenses depletion [2]. The latter was visible through the diminution of levels of vitamin C, β-carotene and uric acid in smokers versus non-smokers. These results support the hypothesis that uric acid acts as an antioxidant in plasma. Actually, some evidence indicates that uric acid may function either as an antioxidant (mainly in plasma) or pro-oxidant (mainly within the cell) [30]. Oxidative degradation of lipids (lipid peroxidation) has been shown to be higher in smokers compared with non-smokers [18,31]. It was here demonstrated by the measurement of MDA level that was higher in smokers. Oxidative modification of LDL can also be evaluated through the measurement of anti-oxLDL antibodies concentration, reported increased in smokers [16]. However, the results obtained in this study shown reduced levels in smokers. It could be explained by a protective role of anti-oxLDL antibodies against oxLDL. Indeed, the role and the interpretation of these antibodies are still controversial in the literature. On one hand, titers of anti-oxLDL antibodies were found to be correlated with the extent of CVDs and, on the other hand, experimental data have indicated that they may neutralize oxLDL, thereby reducing the incidence of CVDs [32]. Although the concentration of iron, a prooxidant, was not shown different when comparing smokers and non-smokers, levels of ferritin and transferrin, two key proteins in its homeostastis were found impacted. As in another study [33], ferritin concentration was shown higher in smokers. It was suggested that expression of this protein was increased in case of iron-catalysed oxidative stress in order to prevent the generation of ROS and free radicals via iron sequestration [33].

Cigarette smoking is classically associated with lipids modifications. Triglycerides level was significantly higher in smokers and thus confirms the literature [34,35]. Interestingly, level of sdLDL which have been associated with an increased risk of CVDs were found lower in smokers than in non-smokers. This observation supports the study of Urahama et al. (2008) that also related this surprising result [36]. The causal mechanism is, however, not yet understood.

Overall, results described above were in agreement with the endothelial dysfunction-related processes reported in the literature. Therefore, the data could be trusted and used to evaluate the possibility of discriminating smokers from non-smokers. For the first time to our knowledge, the features of discrimination of biomarkers other than tobacco were evaluated. As expected, tobacco biomarkers allowed the best discrimination between the two groups with sensitivity and specificity above 88%. Anyway, a sensitivity >81% was achieved for IL-8, ferritin, and transferrin and a specificity >80% was observed for MDA and vitamin C. Given that none of the studied biomarkers allowed both good sensitivity and specificity, combination by logistic regressions was evaluated. The model including vitamin C, ApoB, HbA1c, GPT, and GGT was associated with a high area under the ROC curve (0.877) and allowed 88.5% of correct classifications. Another model including only routine parameters, hsCRP, uric acid, transferrin, HDL, glycemia, GOT, GGT, and albumin, was identified. It was also associated with a good area under the ROC curve (0.928) and allowed 84.3% of correct classifications. This result may open the prospect of assessing patients' tobacco use based on classic parameters of blood test, without the need of specific equipment for medical laboratories. Further work is needed to validate these two models.

A first limitation of this study was that the biomarkers concentrations were measured only once at baseline. Therefore, observed differences between smokers and non-smokers could be due to biological variation, even though statistically significant. Data about biological variation are not available for each parameter, but the differences were still analysed taking into account the variability of analytical tests. The differences were higher than the assays imprecision. A second limitation was the inclusion of COPD patients among smokers, which could affect the visible differences between smokers and non-smokers. Indeed, COPD has been linked to endothelial dysfunction and increased oxidative stress [37]. Since the major environmental risk factor for COPD is inhalation of cigarette smoke [37,38], the recruitment of smokers in real conditions implied the presence of COPD patients. However, participants with acute exacerbations of COPD were excluded. A second comparison between smokers and non-smokers was still carried out excluding COPD patients (128 smokers versus 83 non-smokers). No difference in significance was found. Finally, a third limitation concerns the

models obtained from logistic regressions that have to be validated before considering their use.

2.1.6 Conclusion

Cigarette smoking is associated with endothelial dysfunction, the first step in the development of CVDs. This research evaluated the effect of tobacco smoke in endothelial dysfunction through the study of 138 smokers and 83 non-smokers for which an extensive panel of biomarkers of endothelial function and related processes, such as inflammation, oxidative stress, and lipid profile, were measured. Inflammation was shown increased in smokers through the elevation of IL-8 and hsCRP levels. Concentrations of adhesion molecules, sVCAM and sICAM, were also increased, indicating an increase of the adherence of endothelial cells to immunity cells. Oxidative stress in smokers were reflecting by the depletion of antioxidants (vitamin C, β-carotene, uric acid), the increase of lipid peroxidation (MDA, anti-oxLDL antibodies) and the disruptions of proteins involved in the homeostasis of the pro-oxidant iron (ferritin, transferrin). Lipid profile was also impacted given that elevation of triglycerides and diminution of sdLDL concentrations were observed in smokers. The data were used to determine for the first time to our knowledge the possibility of evaluating the individuals' tobacco status with biomarkers different from the usual tobacco biomarkers. A relation including vitamin C, ApoB, HbA1c, GPT, and GGT allowed to classify correctly 88.5% of cases. Another one including hsCRP, uric acid, transferrin, HDL, glycemia, GOT, GGT, and albumin (only routine parameters) allowed to classify correctly 84.3% of cases. Further work is needed to validate these two models.

2.2 Evaluation of anatabine and anabasine in discriminating smokers and non-smokers

Anatabine and anabasine as specific biomarkers of smoking cessation: time to update the cutoff values?

Paper submitted for publication

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2.2.1 Abstract

Objectives: Anatabine and anabasine are two tobacco alkaloids used to discriminate between tobacco users and abstinents, including users of nicotine replacement therapy. A study determined cutoff values (>2 ng/ml) in 2002, which were not revised since. However, these values seem to be too high, leading to a high likelihood of misclassification: many smokers are wrongly considered abstinent, which results in major consequences, especially increased adverse outcomes of transplantation if smokers are included. The aim of this study is to propose more adequate thresholds to better distinguish tobacco users from non-users and thereby improve patients care.

Design and Methods: A new and more sensitive analytical method by liquid chromatography-mass detection was developed to allow the quantification of low concentrations. Anatabine and anabasine were measured in urines of 116 self-reported smokers and 47 long-term non-smokers (confirmed by the analysis of nicotine and its metabolites). The best compromise between sensitivity and specificity allowed us to determine new cutoff values.

Results: The thresholds >0.097 ng/ml for anatabine and >0.236 ng/ml for anabasine were associated with a sensitivity of 97% (anatabine) and 89% (anabasine) and a specificity of 98% for both alkaloids. These cutoff values greatly increased the sensitivity given that it dropped to 75% (anatabine) and 47% (anabasine) when using the reference value (>2 ng/ml).

Conclusions: The cutoff values >0.097 ng/ml for anatabine and >0.236 ng/ml for anabasine appear to better differentiate tobacco users from abstinents than the current reference threshold (>2 ng/ml for both alkaloids). It may impact considerably patients care, especially in transplantation settings in which smoking abstinence is essential to avoid adverse outcomes of transplantation.

2.2.2 Introduction

Anatabine and anabasine, minor tobacco alkaloids, were proposed as biomarkers to differentiate smokers from non-smokers. Since they are not nicotine-derived and present only in negligible amounts in pharmaceutical grade nicotine, they are especially useful to distinguish between tobacco and nicotine replacement therapy (NRT) users [6,7].

Jacob et al. defined in 2002 the cutpoint of 2 ng/ml for both alkaloids in urine as the criterion to differentiate tobacco users and non-users [7]. The method of quantification (gas chromatography coupled with mass spectrometry) used in their study and developed in 1993 showed a limit of quantification (LOQ) of 1 ng/ml [39]. Moyer et al. (2002) also suggested the use of the threshold >2 ng/ml for anabasine (anatabine not studied), based on a liquid chromatography-tandem mass spectrometry (LC-MS/MS) procedure with LOQ = 1 ng/ml [24]. In 2014, Suh-Lailam et al. used a LC-MS/MS method with LOQ = 2 ng/ml for anabasine (anatabine not studied) to propose the cutoff of 3 ng/ml [40]. It appears that the LOQ of these methods are relatively high compared to the cutoff values, leading generally to unquantifiable concentrations of alkaloids for supposed non-users.

Since then several assays by LC-MS/MS have been developed allowing quantification of lower concentrations: LOQ = 0.15 ng/ml [41], 0.4 ng/ml [42] for anatabine and 0.2 ng/ml [41], 0.5 ng/ml [42] for anabasine. Despite the emergence of this new quantification methods, the cutoff >2 ng/ml established in 2002 by Jacob et al. remains the reference to differentiate between tobacco users and abstinents [6,8,41,43]. However, several studies have drawn attention to a lack of clinical sensitivity when using these biomarkers with such cutoff values: 14% [8], 16% [41] or 60% [9] of self-reported active smokers would have been wrongly considered abstinent due to concentrations of these alkaloids below the cutoff and/or undetectable concentrations by the analytical procedure used. This high likelihood of misclassification may have major consequences, including inappropriate insurance premiums or eligibility for organs transplantation (thereby increasing adverse outcomes of transplantation if smokers are included) [9].

The aim of our study was to propose more adequate cutoff values of anatabine and anabasine to improve the differentiation between tobacco users and non-users by using a new and more sensitive analytical method.

2.2.3 Materials and Methods

One hundred and sixteen self-reported smokers (55 men and 61 women aged 26 to 81 years) and forty-seven long-term non-smokers (25 men and 22 women aged 21 to 82 years) were recruited in the Centre Hospitalier Universitaire of Catholic University of Louvain (Namur, Belgium). Smokers had a median consumption of 20 cigarettes per day (interquartile range: 10-25) and expired monoxide carbon (CO) levels higher than 5 ppm. Informed consent was provided from all participants. The project was approved by the hospital ethics committee and was led according to the Declaration of Helsinki and the Good Clinical Practice guidelines [19].

Urines from smokers and non-smokers were collected to measure anatabine and anabasine. Other tobacco biomarkers – nicotine, cotinine, and trans-3'-hydroxycotinine (3HC) – were measured to objectively confirm the participants' smoking status. The five biomarkers were quantified simultaneously by an in-house-developed method (liquid chromatography-mass detection) that was previously published [20]. This method allowed quantification of low concentrations with an LOQ = 0.029 ng/ml for both anatabine and anabasine.

The tobacco biomarkers comparison in smokers versus non-smokers was carried out by Wilcoxon rank sum test. Differences were considered significant when $P<0.05$. Cutoff values to distinguish the two groups were defined according to the receiver operating characteristic (ROC) curves. Sensitivity (Se), specificity (Sp), positive (PPV) and negative predictive values (NPV) were assessed. PPV and NPV were calculated based on a prevalence of smoking of 20% [22]. All statistical analysis were performed using MedCalc® (version 20.109, MedCalc Software Ltd, Ostend, Belgium).

2.2.4 Results

Table 1 shows expired CO level, urinary measurement of nicotine and its metabolites, and urinary measurement of anatabine and anabasine in the two groups.

Table 1: Median concentrations of tobacco biomarkers in smokers versus non-smokers

	Smokers Median (Interquartile range)	**Non-smokers** Median (Interquartile range)	**P**
CO (ppm)	18 (13 – 23)	-	-
Nicotine (µg/ml)	0.58 (0.23 – 1.11)	0.06 (0.06 – 0.06)	<0.0001
Cotinine (µg/ml)	1.21 (0.85 – 1.71)	0.01 (0.01 – 0.01)	<0.0001
3HC (µg/ml)	3.72 (1.58 – 6.07)	0.02 (0.02 – 0.02)	<0.0001
Anatabine (ng/ml)	5.595 (2.013 – 11.286)	0.029 (0.029 – 0.029)	<0.0001
Anabasine (ng/ml)	1.785 (0.559 – 7.440)	0.029 (0.029 – 0.137)	<0.0001

CO, carbon monoxide; 3HC, trans-3'-hydroxycotinine.

Based on the ROC curves, the cutoff values were >0.097 ng/ml for anatabine and >0.236 ng/ml for anabasine. These new cutoffs were the best compromise between sensitivity and specificity. Table 2 shows the diagnostic features when using these ones in comparison with the cutoffs of reference (>2 ng/ml).

Table 2: Sensitivity, specificity, and predictive values for discrimination of smokers and non-smokers depending on the cutoff values used for anatabine and anabasine.

	Anatabine		**Anabasine**	
Cutoff (ng/ml)	>0.097	>2	>0.236	>2
Se (%)	97	75	89	47
Sp (%)	98	98	98	100
PPV (%)	92	90	91	100
NPV (%)	99	94	97	88

Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value

2.2.5 Discussion

Anatabine and anabasine are two tobacco alkaloids proposed to differentiate smokers from non-smokers. They are absent in nicotine-containing medications given that they are not nicotine-derived. For this reason, these two alkaloids are especially of interest to discriminate between tobacco and NRT users [6,7]. This discrimination is crucial for insurance premiums but also for patients care: follow-up of patients in cessation or eligibility for organs transplantation [9]. The reference cutoff values (>2 ng/ml for both anatabine and anabasine) were determined in 2002 by Jacob et al. [7] and were not revised since. However, several studies have point the poor clinical sensitivity out and put in doubt the utility of these biomarkers without updated cutoff values [8,9,41].

In order to propose more adequate cutoffs for anatabine and anabasine, we developed a new and more sensitive analytical method resulting in an LOQ of 0.029 ng/ml for both anatabine and anabasine [20]. This method allowed the quantification of lower concentrations than the one used in the study of Jacob et al. (2002) (LOQ of 1 ng/ml) which led usually to unquantifiable concentrations for supposed non-users [7,39]. We measured anatabine and anabasine, but also CO, nicotine and its metabolites, in 116 self-reported smokers and 47 long-term non-smokers. Median concentrations of CO, nicotine, cotinine, and 3HC confirmed the participants' smoking status according to the cutoff values proposed in the literature to differentiate smokers from non-smokers: >5 ppm for CO, >0.1 µg/ml for nicotine, >0.073 µg/ml for cotinine, and >0.4 µg/ml for 3HC [6,24].

According to our results, we propose here the cutoff values of >0.097 ng/ml for anatabine and >0.236 ng/ml for anabasine. These new cutoffs were associated with a sensitivity of 97% (anatabine) and 89% (anabasine) and a specificity of 98% for both alkaloids. While using the cutoff of reference (>2 ng/ml), the sensitivity dropped to 75% (anatabine) and 47% (anabasine) with specificity of 98% (anatabine) and 100% (anabasine). This result confirms the lack of clinical sensitivity associated with the reference cutoffs, as reported in other studies [8,9,41].

2.2.6 Conclusion

We propose using the cutoff values of >0.097 ng/ml for anatabine and >0.236 ng/ml for anabasine that may better distinguish tobacco users from non-users than the current reference cutoffs (>2 ng/ml for both anatabine and anabasine). This could impact considerably patients care, especially in transplantation settings in which smoking abstinence is essential to avoid adverse outcomes of transplantation.

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Chapter III. Effect of smoking cessation in endothelial dysfunction

While smoking is admitted as a risk factor for cardiovascular diseases (CVDs) [1,2], it is important to evaluate the possibility of risk decrease on quitting. This information could encourage smokers to enter a cessation program. To date, it is reported that quitting smoking reduces the risk of developing diseases [3], but the implied mechanisms are still unclear [4]. In this chapter, we aim to clearly assess the risk decrease by monitoring biomarkers of endothelial function, oxidative stress, inflammation and lipids on quitting.

We followed the biomarkers concentrations in cigarette smokers at baseline (as studied in the chapter II) and when becoming abstinent. This abstinence was achieved with counseling at the tobaccology unit of the CHU (Centre Hospitalier Universitaire) UCL (Catholic University of Louvain) Namur and, sometimes, medication (nicotine replacement therapy (NRT) or varenicline). The abstinence duration was variable depending on the participants since this study was performed under real conditions: the ability to quit depends on the individual's motivation and his degree of dependence on cigarettes [5]. The median of abstinence was 70 days.

Results obtained can objectify the CVDs risk decrease on quitting given that we showed the decrease of inflammation, endothelium activation, and oxidative stress, each being involved in endothelial dysfunction, the first step of CVDs [1,2]. We also found an improvement of the lipid profile, despite the weight gain associated with the cessation. In view of the abstinence median, this study has shown that some adverse effects of smoking could be reversible even at short-term after quitting. This observation could be a motive for smoking cessation.

3.1 Changes in endothelial dysfunction-related biomarkers concentration on quitting

Changes in biomarkers of endothelial function, oxidative stress, inflammation and lipids after smoking cessation: a cohort study

Paper submitted for publication

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3.1.1 Abstract

Background: Tobacco use is known to be involved in the development of cardiovascular diseases, which leads to premature mortality. Endothelial dysfunction, the first step in this process, was shown induced by smoking. It is reported that quitting smoking could reduce the risk of diseases, but the implied mechanisms are still unclear. This study aimed to evaluate the biological markers of endothelial function in smokers when actively smoking and after cessation.

Methods: Quantification of several biomarkers reflecting inflammation, endothelium activation, oxidative stress, and lipids was performed in sixty-five smokers when actively smoking and after cessation (median abstinence duration of seventy days).

Results: A possible decrease of inflammation was observed through the concentration reduction of a proinflammatory cytokine (interleukine-6) on quitting. A decrease of endothelium activation was visible by the reduced level of the soluble intercellular adhesion molecule. Two antioxidants, uric acid and vitamin C, were found at higher concentration than before the cessation, potentially reflecting the decrease of oxidative stress on quitting. Lipid profile was improved post-quit since HDL level was increased and LDL level was decreased. All these effects were visible at short term with abstinence duration less than seventy days. No sex-specific difference was observed and no additional changes were observed for longer abstinence duration.

Conclusion: These observations suggest that some adverse effects of smoking on endothelial function could be reversible on quitting smoking. It could encourage smokers to enter a cessation program to reduce the risk for cardiovascular diseases development.

3.1.2 Introduction

Tobacco use is associated with four main groups of diseases: cardiovascular diseases (CVDs), cancers, diabetes, and respiratory diseases [6]. Although it is well known that it is one of the leading causes of premature mortality, 22% of the global population still use tobacco [7]. Promoting tobacco cessation is crucial to decrease the health consequences [8].

Approximately 35-40% of smoking-related deaths are due to CVDs. Toxic compounds from inhaled cigarette smoke reach the blood circulation from alveoli and cause endothelial cells activation. It results in a proinflammatory, prothrombotic, and vasoconstrictive state with altered expression of adhesion molecules, platelet activation, enhanced oxidative stress, and impaired vascular tone due to reduced nitric oxide bioavailability [1,9,10]. These processes initiate endothelial dysfunction, which is an early precursor of CVDs [11]. Different studies have indeed shown an increase of inflammation, adhesion molecules level, and oxidative stress as well as altered lipid profile in smokers compared with non-smokers [2,12–14]. It was reported that quitting smoking reduces the risk of developing diseases [3] and that smoking-induced endothelial dysfunction could be reversible on quitting. However, the mechanisms implied in the CVDs risk decrease after smoking cessation are still poorly documented [4].

The study of endothelial dysfunction-related biomarkers in smokers becoming abstinent could objectify the risk decrease. The most common biomarker used is the arterial brachial flow-mediated dilation (FMD) that allows to show reduced vasodilative response to endothelial stimuli. However, this measurement is dependent on physiological and operator variability, therefore, other endothelial biomarkers have been proposed. For example, levels of asymmetric dimethylarginin (ADMA) or proinflammatory molecules (VCAM, ICAM, selectins, CRP, TNF-α) can be evaluated. Recently, novel biomarkers have been suggested for combination with traditional biomarkers to enhance cardiovascular prevention. However, they are not yet implemented in routine screening [15]. Previous studies have compared several biomarkers concentration in active smokers and former smokers and have shown an improvement of endothelial function in the latters [16–18]. For example, through the quantification of endothelial-coagulative biomarkers, Caponnetto et al. has suggested the reversal of endothelial-coagulative abnormalities in smokers by quitting smoking [19]. However, many biomarkers linked to inflammation, oxidative stress, and lipids are still not studied and short-term effects of smoking cessation remain poorly studied. The aim of the present study was to follow the concentration of biomarkers of endothelial function, inflammation, oxidative stress, and lipids in smokers when actively

smoking and after smoking cessation. Results were also compared to levels observed in non-smokers in our previous study [14]. Moreover, the influence of the duration of smoking abstinence on biomarkers changes was evaluated.

3.1.3 Materials and Methods

Study design

This study is a follow-up to our previous study [14] comparing endothelial dysfunction-related biomarkers in smokers and non-smokers. The present study was designed in order to evaluate the biomarkers concentration changes in smokers who stopped smoking. The biomarkers were quantified for all smokers who entered a smoking cessation program. Then, participants had to declare their quit date by phone call. During the follow-up, only smokers who successfully quit were selected for the evaluation of biomarkers concentration changes after continuous abstinence. Therefore, biomarkers were quantified at baseline and post-quit (Figure 1). To be eligible, participants had to become abstinent (0 cigarette per day) for at least a uninterrupted period of three weeks between the baseline visit ("visit 0") and the follow-up visit ("visit 1"). Smokers who relapsed before were not included in the study. Reporting of the study conforms to broad EQUATOR guidelines [20].

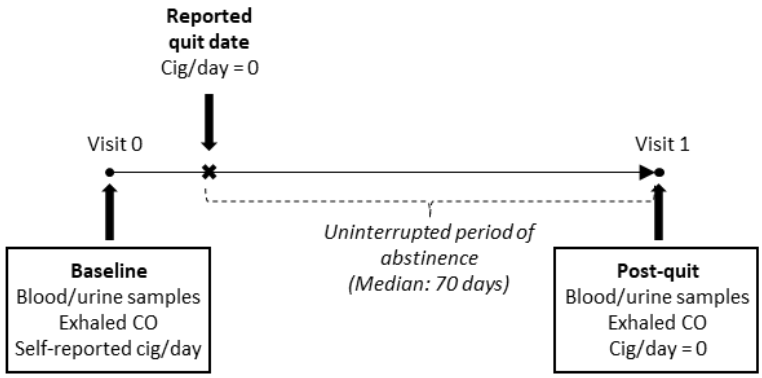

Figure 1: Design of the study. Smokers included in the study entered a smoking cessation program. Biomarkers in blood/urine samples were measured at baseline (visit 0). The active smoking status was assessed by the self-reported consumption of cigarettes per day, exhaled carbon monoxide (CO), and five tobacco biomarkers. Participants reported their quit date by phone call. Biomarkers were then measured post-quit (visit 1) after a uninterrupted period of abstinence, which depended on the participant (median: 70 days). Abstinence was assessed by the self-reported consumption, exhaled CO, and five tobacco biomarkers. Smokers who relapsed before the minimal period of three weeks were not included in the study.

Setting

Smokers were recruited at the tobaccology unit of the Centre Hospitalier Universitaire (CHU) of Catholic University of Louvain (UCL) (Namur, Belgium) from February 2018 to July 2021. Follow-ups ended in October 2021.

Subjects

Participants were 65 cigarette smokers, 33 men and 32 women aged 26 to 80 years, recruited on a voluntary basis during our previous study [14]. Three of them suffered from chronic obstructive pulmonary disease (COPD) without acute pathology. None suffered from cardiovascular diseases. Each participant entered a smoking cessation program: thirty-five used Nicotine replacement therapy (NRT) products (gums, transdermal patches, oral sprays, or inhalers), 22 resorted to varenicline (Champix®), and 8 quit without medication.

The median smoking abstinence duration was 70 days (interquartile range: 45 – 105 days) with a minimal duration of 21 days and a maximal duration of eight months. Informed written consent was obtained from all participants. The project was approved by the CHU UCL Namur's ethics committee and was led according to the Declaration of Helsinki and the Good Clinical Practice guidelines [21].

Smoking cessation program

The smoking cessation program was led following the clinical practice guidelines on smoking cessation of the Agency for Health Care Policy and Research [22]. At the baseline visit, the tobacco consumption was evaluated by self-report, which allowed the calculation of the number of packs per year, and the measurement of exhaled carbon monoxide (CO) and five tobacco alkaloids (nicotine, cotinine, trans-3'-hydroxycotinine (3HC), anatabine and anabasine). Nicotine dependence level was assessed by the Fagerström test [23]. Participants received psychological support and medications for quitting. The treatment was personalised depending on contraindications and participants' preference, with NRT or varenicline in first line. Follow-up visits were left to the discretion of the smokers, by phone call or face-to-face support. The quit date was not standardised, but participants had to declare this date by phone call. At the follow-up visit (visit 1), they confirmed their complete abstinence since the quit date and samples were collected. Only patients with complete abstinence for at least three weeks were evaluated.

Biospecimen collection

Blood samples and urine were collected twice: at baseline when actively smoking (visit 0) and post-quit (0 cigarette per day) with or without treatment (visit 1). Samples were treated similarly for the two visits. Several aliquots (urine, heparin whole blood, and EDTA whole blood) were immediately frozen at -20°C for later batch analyses. Serum, sodium fluoride plasma and heparinised plasma were collected after a centrifugation (15 minutes, 5±3°C, 5950 g, SL16R, Thermo Scientific, USA) to directly perform some analyses. Aliquots of plasma and serum were also frozen at -20°C for chromatographic assays and enzyme linked immunosorbent assays (ELISA).

Quantitative instruments

Quantification of some parameters on fresh centrifuged blood samples were performed using VITROS® 5600 System and VITROS® 3600 System from Ortho Clinical Diagnostics (Raritan, USA).

Two chromatographic systems were used. The first one was a high-performance liquid chromatography (HPLC) system coupled with an ultraviolet (UV) detector (Alliance 2695, detector 2489, Waters, Milford, USA). The second one was an ultra-high performance liquid chromatography (UHPLC) system (Acquity UPLC® H-Class, Waters), coupled with a photodiode array detector (Acquity PDA), a fluorescence detector (Acquity FLR), or a mass detector (Acquity QDa). Both systems were associated with Empower 3 (Waters) to acquire and process data. Runs were launched in batch and calibration of systems were performed each day of use.

ELISA assays required a microplate strip washer (ELx50, Agilent, Santa Clara, USA) and an absorbance microplate reader (800TS, Agilent). These tests were performed in batch.

Other analyses were carried out in batch using the BN ProSpec® System (Siemens Healthcare, Saint-Denis Cedex, France).

Biomarkers concentrations determination

Tobacco exposure biomarkers

The exhaled CO concentration was measured by electrochemistry with a Smokerlyzer® monitor (Bedfont Scientific Ltd, Harrietsham, UK). Nicotine and its metabolites – cotinine and 3HC – were analysed to monitor the participants' smoking status. Anatabine and anabasine were analysed to objectively confirm the tobacco abstinence [24,25]. These five alkaloids were quantified in urine simultaneously by UHPLC-mass detection [26].

Inflammatory biomarkers

All studied inflammatory biomarkers were analysed in serum. High-sensitivity C-reactive protein (hsCRP) was quantified by nephelometry with the BN ProSpec. ELISA kits from Abcam B.V. (Amsterdam, Netherlands) were used to measure concentrations of monocyte chemoattractant protein 1 (MCP-1) and interleukines (IL) 6, 8, and 10. An ELISA kit from Tecan (IBL International GmbH, Hamburg, Germany) allowed to assess the tumor necrosis factor alpha (TNF-α) concentration.

Endothelial function biomarkers

Asymmetric dimethylarginine (ADMA), soluble vascular cell adhesion molecule (sVCAM), and soluble intercellular adhesion molecule (sICAM) were measured in serum using ELISA kits from MT-Diagnostics B.V. (Etten-Leur, Netherlands).

Oxidative status biomarkers

VITROS 5600 was used to determine iron and uric acid in serum by colorimetry, transferrin in serum by turbidimetry (reagent pack TFTUR-C00, DiAgam, Ghislenghien, Belgium), glutathione peroxidase (GPX) in heparin whole blood through an enzymatic method (kit RANSEL, Randox, Crumlin, UK), and superoxide dismutase (SOD) in EDTA whole blood through a colorimetric activity kit (kit RANSOD, Randox, Crumlin, UK). VITROS 3600 was used to measure ferritin in serum by immunoassay.

Respective Chromsystems (Munich, Germany) kits were used to quantify vitamins A/C/E, malondialdehyde in plasma, and coenzyme Q10, β-carotene in serum. All were detected by HPLC-UV, except for malondialdehyde and vitamins A/E that were detected in UHPLC. Separation was performed on a reverse phase column (Luna Omega 1.6 µm C18 100Å 100X2.1 mm, Phenomenex, Torrance, USA) with a flow rate of 0.3 ml/min followed by a fluorescence detection (excitation wavelength: 515 nm; emission wavelength: 553 nm) for malondialdehyde. Vitamins A/E were analysed on a reverse phase column (Acquity UPLC HSS T3 1.8 µm 2.1 X 50 mm, Waters) with a flow rate of 0.6 ml/min and photodiode array detection (scan 210-400 nm).

Anti-oxidized low-density lipoprotein (anti-oxLDL) antibodies were quantified in serum by an ELISA kit (Biomedica Medizinprodukte GmbH, Wien, Austria).

Lipids

VITROS 5600 was used to assess cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride levels in serum by enzymatic methods. BN ProSpec was used to quantify lipoprotein (a) (Lp(a)), apolipoprotein (Apo) A1, and ApoB by nephelometry. Small dense LDL cholesterol (sdLDL) concentration was measured with an ELISA kit (Cubasio, Houston, USA).

Statistical analyses

Non-parametric tests were used given that the normality of the data was rejected by Shapiro-Wilk test. Results were expressed by median and interquartile range. The participants' biomarkers comparison at visit 0 (actively smoking) versus at visit 1 (post-quit) was performed by Wilcoxon signed rank test. Associations between biomarkers concentration at visit 0 and the number of packs (20 cigarettes/pack) per year were addressed by Spearman correlation coefficients. Comparison of biomarkers concentration changes between the two visits (visit 1 – visit 0) for men and women was performed by Wilcoxon rank sum test. The same test was used to evaluate the effect of abstinence duration on biomarkers concentration changes in participants with less than 70 days of abstinence (group 1) versus participants with more than 70 days of abstinence (group 2). Participants with exactly 70 days of abstinence (n=7) were excluded of this comparison. The impact of this exclusion was checked by testing the two other cases: adding these participants to the group 1 or to the group 2. No difference in significance was found. For all analyses, differences were considered significant when $P<0.05$. Tests were performed using MedCalc® (version 20.109, MedCalc Software Ltd, Ostend, Belgium).

3.1.4 Results

Smoking status monitoring

The smoking status of enrolled participants was monitored at baseline (visit 0) and after quitting smoking (visit 1) by the self-reported number of cigarettes per day and the measurement of exhaled CO and five urinary tobacco alkaloids (nicotine, cotinine, 3HC, anatabine, and anabasine). Results are shown in Table 1.

Table 1: Medians of tobbaco biomarkers in smokers at baseline (active smoking) and post-quit (minimum 21 days of abstinence)

	Baseline Median (Interquartile range) (n=65)	**Post-quit** Median (Interquartile range) (n=65)	**P**
Number of cigarettes per day	20 (10 – 25)	0 (0 – 0)	<0.0001
CO (ppm)	18 (13 – 25)*	2 (1 – 2)*	<0.0001
Nicotine (µg/ml)	0.612 (0.267– 1.006)*	0.055 (0.055 – 0.235)*	<0.0001
Cotinine (µg/ml)	1.16 (0.83 – 1.63)	0.15 (0.01 – 0.88)	<0.0001
3HC (µg/ml)	3.51 (1.65 – 6.03)*	0.41 (0.02 – 2.27)*	<0.0001
Anatabine (ng/ml)	5.803 (2.206 – 11.057)*	0.029 (0.029 – 0.071)*	<0.0001
Anabasine (ng/ml)	1.866 (0.582 – 8.062)*	0.032 (0.029 – 0.198)*	<0.0001

CO, carbon monoxide; 3HC, *trans*-3'-hydroxycotinine. *n=54

Changes in biomarkers involved in the endothelial function on quitting smoking

Inflammatory biomarkers

A significant decrease in the IL-6 concentration was found when quitting smoking (Table 2). The values distributions at baseline and post-quit was significantly different for TNF-α concentration although the medians were identical. A slight decrease, nearly significant, in hsCRP level when stopping was observed. No other changes were observed.

Table 2: Median concentrations of inflammatory biomarkers in smokers at baseline (active smoking) and post-quit (minimum 21 days of abstinence)

	Baseline Median (Interquartile range) (n=65)	**Post-quit** Median (Interquartile range) (n=65)	**P**
IL-6 (pg/ml)	3.1 (2.0 – 4.5)	2.5 (1.8 – 3.8)	0.0104
IL-8 (pg/ml)	9.2 (6.2 – 14.6)	9.1 (5.7 – 13.4)	0.5167
IL-10 (pg/ml)	0.4 (0.4 – 1.5)	0.7 (0.4 – 1.7)	0.2150
TNF-α (pg/ml)	0.15 (0.15 – 0.41)	0.15 (0.15 – 0.32)	0.0163
MCP-1 (pg/ml)	75.5 (53.1 – 146.5)	86.8 (53.3 – 134.6)	0.5412
hsCRP (mg/l)	1.8 (0.8 – 3.3)	1.4 (0.8 – 2.4)	0.0924

IL, interleukine; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; hsCRP, high-sensitivity C-reactive protein

Endothelial function biomarkers

When comparing levels of endothelial biomarkers at baseline and post-quit, only sICAM showed a concentration change: it was lower when becoming abstinent (Table 3).

Table 3: Median concentrations of biomarkers of endothelial function in smokers at baseline (active smoking) and post-quit (minimum 21 days of abstinence)

	Baseline Median (Interquartile range) (n=65)	**Post-quit** Median (Interquartile range) (n=65)	**P**
ADMA (µmol/l)	0.6 (0.5 – 0.8)	0.6 (0.5 – 0.8)	0.3434
sVCAM (ng/ml)	788 (655 – 1025)	860 (662 – 1035)	0.8574
sICAM (ng/ml)	356 (241 – 438)	301 (142 – 428)	0.0022

ADMA, asymmetric dimethylarginine; sVCAM, soluble vascular cell adhesion molecule; sICAM, soluble intercellular adhesion molecule

Oxidative stress biomarkers

Table 4 shows that few oxidative stress biomarkers were affected by smoking cessation. However, uric acid concentration was significantly higher after quitting than at baseline. The vitamin C concentration tended towards increase after quitting (almost significant).

Table 4: Median concentrations of biomarkers of oxidative status in smokers at baseline (active smoking) and post-quit (minimum 21 days of abstinence)

	Baseline Median (Interquartile range) (n=65)	**Post-quit** Median (Interquartile range) (n=65)	**P**
SOD (U/ml)	257.5 (225.2 – 282.3)	244.0 (208.2 – 272.0)	0.0932
GPX (U/l)	12234 (9852 – 15261)*	12435 (10667 – 14619)*	0.5376
MDA (µg/l)	7.2 (5.5 – 10.9)*	6.8 (5.6 – 9.1)*	0.1620
Uric acid (mg/dl)	4.8 (4.0 – 5.9)	5.4 (4.4 – 6.2)	0.0001
Iron (µg/dl)	105 (74 – 122)	101 (84 – 124)	0.4829
Ferritin (ng/ml)	79.1 (37.4 – 131.5)	76.3 (39.0 – 139.0)	0.7205
Transferrin (g/l)	2.51 (2.29 – 2.85)**	2.49 (2.24 – 2.78)**	0.6755
Vitamin A (mg/l)	0.53 (0.42 – 0.63)	0.54 (0.45 – 0.63)	0.2474
Vitamin E (mg/l)	12.9 (10.5 – 16.9)	13.6 (10.8 – 15.4)	0.4084
Vitamin C (mg/l)	9.5 (7.6 – 12.6)	10.8 (8.5 – 13.8)	0.0647
Coenzyme Q10 (µg/l)	827 (601 – 1083)	859 (613 – 1133)	0.5499
β-carotene (ng/ml)	159 (107 – 270)	166 (104 – 288)	0.9557
Anti-oxLDL (U/l)	162 (84 – 289)	156 (70 – 400)	0.1498

SOD, superoxide dismutase; GPX, glutathione peroxidase; MDA, malondialdehyde; anti-oxLDL, anti-oxidized LDL antibodies. *n=33 **n=39

Lipid profile

Several modifications were observed in the lipid profile of smokers when stopping (Table 5). However, it should be noticed that participants' weight gain was observed between the two visits: medians (interquartile ranges) are 73 (65 – 86) kg for visit 0 versus 75 (66 – 87) kg for visit 1. HDL, ApoA1, and ApoB were at higher concentrations post-quit than at baseline. LDL and sdLDL concentrations were lower than at baseline.

Table 5: Median concentrations of lipids in smokers at baseline (active smoking) and post-quit (minimum 21 days of abstinence)

	Baseline Median (Interquartile range) (n=65)	**Post-quit** Median (Interquartile range) (n=65)	**P**
Cholesterol (mg/dl)	198 (170 – 227)	198 (173 – 227)	0.6882
HDL (mg/dl)	52 (42 – 66)	56 (45 – 68)	0.0320
LDL (mg/dl)	124 (92 – 156)	122 (87– 146)	0.0013
Triglycerides (mg/dl)	120 (96 – 169)	141 (92 – 192)	0.8672
Lp(a) (g/l)	0.07 (0.03 – 0.28)	0.09 (0.03 – 0.27)	0.7293
ApoA1 (g/l)	1.55 (1.37 – 1.79)	1.63 (1.43 – 1.87)	0.0114
ApoB (g/l)	0.97 (0.81 – 1.18)	0.99 (0.77 – 1.14)	0.0046
sdLDL (nmol/ml)	1269 (649 – 2987)	1106 (661 – 2582)	0.0270

HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lp(a), lipoprotein (a); Apo, apolipoprotein; sdLDL, small dense LDL

Sex differences

No significant difference was observed when comparing concentration changes in biomarkers of inflammation, endothelial function, oxidative stress, and lipid profile, in men or women (Supplementary table 1).

Influence of the durations of smoke exposure and abstinence on biomarkers concentration

Cotinine, anatabine, IL-6, and sdLDL concentrations at baseline were found significantly correlated to the number of packs per year (Supplementary table 2).

When comparing the biomarkers concentration changes between the two visits (at baseline and post-quit) in participants who were abstinent for less than 70 days (group 1) or more than 70 days (group 2), there were no significant differences, except for IL-6 (Supplementary

tables 3, 4, 5, and 6). However, this observed difference was slight and probably not biologically relevant.

3.1.5 Discussion

Tobacco use is involved in CVDs development by causing endothelial dysfunction. It is associated with increased inflammation and oxidative stress as well as altered lipid profile [1,2]. Endothelial function is reported improved when quitting smoking, which could reduce the CVDs risk [4,16]. An objective method to evaluate the risk decrease is to resort to endothelial dysfunction-related biomarkers and to follow their concentration changes on quitting. We followed a cohort of smokers during their cessation program and we selected a large panel of biomarkers to best address the different mechanisms involved in endothelial dysfunction: endothelial activation was demonstrated through adhesion molecules and nitric oxide bioavailability; inflammation process was shown by pro- and anti-inflammatory cytokines, chemokines, and acute-phase protein; oxidative stress was reflected by prooxidants, enzymes or molecules involved in antioxidant defense, and markers of lipid peroxidation; lipid metabolism was evaluated by the measurement of various lipids. Reported changes in biomarkers were analysed taking into account the variability of analytical tests: all observed changes were higher than coefficients of variation reflecting the assays precision.

The participants' active smoking status at baseline was assessed by the quantification of tobacco exposure biomarkers. A positive correlation was found between cotinine and anatabine and the number of packs per year. The participants' smoking cessation was self-reported and proved by the tobacco biomarkers concentrations that were typical of non-smokers: CO amount was lower than 5 ppm [25], anatabine concentration was lower than 0.097 ng/ml, and anabasine concentration was lower than 0.236 ng/ml (Colsoul et al., 2022). Anatabine and anabasine are two alkaloids not nicotine-derived allowing to prove tobacco abstinence, even in nicotine replacement therapy (NRT) users [24]. As expected, concentrations of nicotine and its metabolites – cotinine and 3HC – were not similar to the ones observed in non-smokers [14,27] due to a part of participants resorted to NRT.

Inflammation is reported higher in smokers than in non-smokers. It is demonstrated, for example, by the elevation of IL-6 concentration, which is a proinflammatory cytokine [28]. The present results showed that chronic exposure to cigarette smoke could contribute to inflammation given that a positive association between the number of packs per year and IL-6 concentration was found. On quitting smoking, decrease of inflammation was observed through the significant reduction of the IL-6 level. Two hypotheses could explain this decrease: smoking-induced inflammation is reduced because of the smoking stop only, or also through the use of NRT in some participants that could help reduce the IL-6 production. Indeed, an anti-inflammatory effect of nicotine was showed in vitro by diminishing IL-6 production [29]. The inflammation decrease was also observed by a slight hsCRP level decrease (almost significant). The circulating level of this acute phase protein is higher in smokers [14,30] and studies have shown that level does not fall immediately upon cessation due to tissue damage that requires time to recover [31]. Some studies mention higher levels of CRP in former smokers than in never-smokers for up to 5 years following cessation [30]. Some others even mention that CRP levels are still raised in former smokers up to 10 to 20 years [31]. Although the median smoking abstinence duration was only 70 days in this study, the post-quit CRP level (median: 1.4 mg/l) was quite similar to the one observed in non-smokers (median: 1.2 mg/l [14]).

The concentration of sICAM, a cell adhesion molecule reflecting the endothelium activation, was decreased after cessation. It confirms results of previous pilot studies also reported a reduction of sICAM induced by smoking cessation [17,32]. The sICAM level in quitters tends to be similar to the level observed in non-smokers (medians: 301 ng/ml in quitters and 328 ng/ml in non-smokers [14]).

Although smoking cessation is reported to decrease oxidative stress, there are few studies addressing this topic [33,34]. In this study, few biomarkers of oxidative stress were found impacted by the cessation, despite the study of a large number of biomarkers that were selected to reflect reactive oxygen species (ROS)-induced damages, ROS generation, and antioxidants. Anyway, uric acid was observed at increased concentration post-quit. It supports its antioxidant role in plasma [35]. Interestingly, the level in quitters were identical

to the one in non-smokers (medians: 5.4 mg/dl [14]). A slight increase of vitamin C concentration, another antioxidant, was also observed (almost significant). These results are consistent with the reported decrease of oxidative stress in abstinents [33,34].

More changes were visible in lipid profile when quitting smoking. This possibly suggests that the concentration of these markers changes at shorter term than other studied endothelial dysfunction-related biomarkers. However, these changes may not only be attributed to smoking cessation, but also to the impact of weight gain associated with the cessation [36]. Indeed, it is known that most people who stop smoking gain weight, which could influence biomarkers, especially the ones reflecting lipid profile [37]. In the present study, an increase of weight was actually observed (medians from 73 kg at baseline to 75 kg after smoking cessation). Forey et al. stipulating that quitting smoking leads to rapid (in less than three weeks) increase of HDL concentration, despite weight gain [38]. Here, an increase of HDL level associated with a decrease of LDL and sdLDL levels were observed. These variables were not adjusted for the weight deliberately since their measurements were more accurate than the latter. This adjustment could wrongly affect the observed effects. The chronic exposure to cigarette smoke appears to be correlated with sdLDL concentration at baseline, which is considered a risk for CVDs [39]. The observed decrease of sdLDL concentration on quitting could, therefore, reflect the risk reduction. Some authors recommend the use of ApoA1 and ApoB as cardiovascular risk marker instead of HDL and LDL [40,41]. As expected, ApoA1 was also found at increased concentration on quitting given that it is the main protein in HDL particles [41]. An unexpected result was observed for ApoB that was at higher concentration post-quit. As ApoB is a part of LDL, a decrease was expected instead. However, ApoB is not only a part of LDL, but also of other potentially atherogenic particles (very LDL, intermediate-density lipoprotein, large buoyant and small dense LDL) [41]. Therefore, this result could possibly be explained by the increase of participants' weight after cessation, impacting largely all atherogenic particles.

While there exist sex-specific differences in risk factors associated with endothelial dysfunction and progression of CVDs [42], no difference was observed in biomarkers concentration changes between the two visits (visit 1 – visit 0) when comparing men and

women. It suggests that there is no sex-specific difference in the potential reversibility of smoking-induced endothelial dysfunction.

In order to evaluate the influence of the smoking abstinence duration, the biomarkers concentration changes were compared in two groups: abstinents for less than 70 days or more than 70 days. It appeared that no differences were visible between the two groups, indicating that even with longer abstinence duration, no additional changes in biomarkers concentrations could be found.

A limitation of this study is the potential influence of treatment for cessation on biomarkers concentration. Some quitters resorted to NRT or varenicline while others quit without medication. This study addressed short-term changes in real conditions of smoking cessation program and, therefore, involved the presence of treatments for some participants. Another study is in progress to evaluate the effect on biomarkers depending on the aid used for smoking cessation.

3.1.6 Conclusion

This research shows that the altered endothelial function in the studied cohort of smokers could be improved by smoking cessation. Inflammation decrease on quitting was visible through the reduction of IL-6 level and the tendency of hsCRP level decrease. The concentration of sICAM, reflecting the endothelium activation, was lower after quitting and tended toward similar concentration than the one observed in non-smokers. Two antioxidants, uric acid and vitamin C (almost significant), were found at higher concentration than before the cessation. Uric acid levels in abstinents and in non-smokers were identical. Concentration of HDL was higher while concentration of LDL was lower after quitting, despite the weight gain associated with the cessation. No influence of the smoking abstinence duration on the biomarkers concentration changes was found. These results emphasise that some adverse effects of smoking could be reversible on quitting, even at short term, contributing to reduce the risk of CVDs development. These results have to be confirmed in a larger population, but the documentation of these early effects could encourage smokers to enter a cessation program.

3.1.7 Supplementary tables

Supplementary table 1: Concentration changes in biomarkers on quitting smoking for men and women.

	Changes in men Median (Interquartile range) (n=33)	**Changes in women** Median (Interquartile range) (n=32)	**P**
Nicotine (µg/ml)	-0.214	-0.255	0.8830
Cotinine (µg/ml)	-0.79	-0.81	0.8562
3HC (µg/ml)	-2.27	-1.71	0.9310
Anatabine (ng/ml)	-9.154	-5.073	0.1459
Anabasine (ng/ml)	-2.231	-0.806	0.0898
IL-6 (pg/ml)	-0.4	-0.5	0.7930
hsCRP (mg/l)	-0.3	0.1	0.0943
sICAM (ng/ml)	-8	-54	0.3315
Uric acid (mg/dl)	0.4	0.3	0.7882
Vitamin C (mg/l)	0.9	0.3	0.6420
HDL (mg/dl)	3	2	0.5327
LDL (mg/dl)	-7	-9	0.3448
ApoA1 (g/l)	0.09	0.03	0.4949
ApoB (g/l)	-0.03	-0.05	0.2563
sdLDL (nmol/ml)	-138	-84	0.6110

3HC, trans-3'-hydroxycotinine; IL, interleukine; hsCRP, high-sensitivity C-reactive protein; sICAM, soluble intercellular adhesion molecule; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo, apolipoprotein; sdLDL, small dense LDL

Supplementary table 2: Association between biomarkers and number of packs per year.

	Spearman coefficient	**P**
Cotinine	0.5	0.0001
Nicotine	0.123	0.3856
3HC	0.0969	0.4945
Anatabine	0.304	0.0286
Anabasine	0.0333	0.8145
IL-6	0.321	0.0140
hsCRP	0.144	0.2816
sICAM	0.166	0.2119
Uric acid	-0.066	0.6228
Vitamin C	-0.116	0.3945
HDL	0.0743	0.5793
LDL	0.0586	0.6621
ApoA1	0.05	0.7094
ApoB	0.0591	0.6596
sdLDL	0.294	0.0278

3HC, trans-3'-hydroxycotinine; IL, interleukine; hsCRP, high-sensitivity C-reactive protein; sICAM, soluble intercellular adhesion molecule; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo, apolipoprotein; sdLDL, small dense LDL

Supplementary table 3: Changes in inflammatory biomarkers concentration of smokers between the two visits (Visit 1 post-quit - Visit 0 at baseline). Abstinence was less than 70 days for group 1 and more than 70 days for group 2.

	Changes in group 1 Median (Interquartile range) (n=30)	**Changes in group 2** Median (Interquartile range) (n=28)	**P**
IL-6 (pg/ml)	-0.2 (-0.7 – 0.8)	-0.9 (-2.2 – -0.3)	0.0085
IL-8 (pg/ml)	0.3 (-1.8 – 1.6)	0.5 (-2.4 – 2.2)	0.7794
IL-10 (pg/ml)	0.0 (-0.4 – 0.5)	0.0 (0.0 – 0.4)	0.3644
TNF-α (pg/ml)	0.0 (-0.1 – 0.0)	0.0 (-0.04 – 0.0)	0.5317
MCP-1 (pg/ml)	0.9 (-26.3 – 18.6)	2.1 (-20.0 – 24.1)	0.7321
hsCRP (mg/l)	-0.01 (-0.53 – 0.43)	-0.36 (-1.68 – 0.18)	0.0735

IL, interleukine; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; hsCRP, high-sensitivity C-reactive protein

Supplementary table 4: Changes in endothelial function biomarkers concentration of smokers between the two visits (Visit 1 post-quit - Visit 0 at baseline). Abstinence was less than 70 days for group 1 and more than 70 days for group 2.

	Changes in group 1 Median (Interquartile range) (n=30)	**Changes in group 2** Median (Interquartile range) (n=28)	**P**
ADMA (µmol/l)	0.06 (-0.05 – 0.13)	0.01 (-0.17 – 0.16)	0.5860
sVCAM (ng/ml)	-15 (-100 – 42)	9 (-119 – 108)	0.5860
sICAM (ng/ml)	-36 (-72 – -1)	-10 (-80 – 57)	0.3668

ADMA, asymmetric dimethylarginine; sVCAM, soluble vascular cell adhesion molecule; sICAM, soluble intercellular adhesion molecule

Supplementary table 5: Changes in oxidative status biomarkers concentration of smokers between the two visits (Visit 1 post-quit - Visit 0 at baseline). Abstinence was less than 70 days for group 1 and more than 70 days for group 2.

	Changes in group 1 Median (Interquartile range) (n=30)	**Changes in group 2** Median (Interquartile range) (n=28)	**P**
SOD (U/ml)	-4.2 (-25.8 – 15.0)	-9.0 (-78.6 – 30.5)	0.7016
GPX (U/l)	-115 (-2801 – 2558)[a]	-955 (-2243 – 1763)[b]	0.8504
MDA (µg/l)	0.27 (-3.57 – 1.21)[c]	-1.09 (-2.90 – 1.02)[d]	0.8129
Uric acid (mg/dl)	0.31 (-0.24 – 0.64)	0.48 (-0.01 – 0.92)	0.1698
Iron (µg/dl)	-8.0 (-34.5 – 22.8)	8.5 (-6.5 – 29.0)	0.1214
Ferritin (ng/ml)	4.8 (-10.6 – 13.3)	-3.0 (-10.3 – 11.8)	0.7254
Transferrin (g/l)	-0.1 (-0.2 – 0.1)[c]	0.0 (-0.1 – 0.2)[d]	0.5254
Vitamin A (mg/l)	0.04 (-0.04 – 0.13)	-0.01 (-0.11 – 0.8)	0.1614
Vitamin E (mg/l)	-0.72 (-2.72 – 2.12)	-0.45 (-3.40 – 2.44)	0.8154
Vitamin C (mg/l)	0.23 (-2.26 – 3.69)	1.24 (-2.03 – 3.85)	0.5552
Coenzyme Q10 (µg/l)	-81.64 (-232.94 – 81.87)	-22.71 (-99.36 – 86.73)	0.1808
β-carotene (ng/ml)	0.68 (-32.72 – 27.87)	-6.93 (-69.48 – 29.53)	0.7204
Anti-oxLDL (U/l)	4.68 (-46.50 – 79.70)	7.10 (-11.97 – 84.83)	0.5123

SOD, superoxide dismutase; GPX, glutathione peroxidase; MDA, malondialdehyde; anti-oxLDL, anti-oxidized LDL antibodies. [a]n=17 [b]n=9 [c]n=19 [d]n=11

Supplementary table 6: Changes in lipids concentration of smokers between the two visits (Visit 1 post-quit - Visit 0 at baseline). Abstinence was less than 70 days for group 1 and more than 70 days for group 2.

	Changes in group 1 Median (Interquartile range) (n=30)	**Changes in group 2** Median (Interquartile range) (n=28)	**P**
Cholesterol (mg/dl)	-6.0 (-16.0 – 7.0)	-3.0 (-16.5 – 17.5)	0.3875
HDL (mg/dl)	2.5 (-5.0 – 7.0)	4.0 (-3.5 – 8.0)	0.4453
LDL (mg/dl)	-8.6 (-15.1 – -4.0)	-7.4 (-21.5 – 6.7)	0.7321
Triglycerides (mg/dl)	2.5 (-20.0 – 52.0)	-1.0 (-44.5 – 51.5)	0.5967
Lp(a) (g/l)	0.00 (-0.03 – 0.02)	0.00 (-0.02 – 0.01)	0.9362
ApoA1 (g/l)	0.07 (-0.03 – 0.17)	0.00 (-0.07 – 0.15)	0.4005
ApoB (g/l)	-0.04 (-0.08 – 0.05)	-0.06 (-0.16 – 0.03)	0.1992
sdLDL (nmol/ml)	-102 (-226 – 56)	-181 (-586 – 104)	0.3302

HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lp(a), lipoprotein (a); Apo, apolipoprotein; sdLDL, small dense LDL

3.2 References of chapter III

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Chapter IV. Effect of the nicotine replacement therapy in endothelial dysfunction

In the chapter III, we showed that some adverse effects of smoking, leading to endothelial dysfunction, could be reversible on quitting. However, some people resort to nicotine replacement therapy (NRT) to quit smoking [1] and, therefore, still use nicotine. The question that remains is "which tobacco component is responsible for endothelial dysfunction?" [2]. If nicotine is responsible for these adverse effects, it could dissuade doctors to prescribe NRT while it greatly increases rates of successful quitting [3].

In this chapter, we are interested in evaluating the impact of NRT on endothelial dysfunction-related biomarkers. To that end, we quantified the biomarkers concentrations in smokers at baseline (see chapter II) and post-quit, with a treatment (NRT or varenicline) or not. The biomarkers concentration changes between the two stages were compared for NRT users and others.

It appears that changes in biomarkers concentration were effectively observed on quitting (see chapter III) but no significant difference was seen in these changes between NRT users and others. These results support the hypothesis that it is another constituent of tobacco than nicotine that is responsible for endothelial dysfunction. They could contribute to the encouragement of prescribing NRT to smokers in order to increase their chances of success in quitting.

4.1 Effect of the nicotine replacement therapy on endothelial dysfunction-related biomarkers on quitting

Effect of the nicotine replacement therapy on biomarkers of inflammation, endothelial dysfunction, oxidative stress and lipids in smokers who quit smoking

Paper submitted for publication

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4.1.1 Abstract

Introduction: The effect of nicotine or nicotine replacement therapy (NRT) on inflammatory processes is controversial and health care professionals may have concerns to prescribe this first line smoking cessation treatment. Therefore, the present study assessed the influence of NRT on biomarkers of inflammation, endothelial function, oxidative stress, and lipids, in smokers who quit smoking.

Methods: Sixty-five cigarette smokers were recruited and followed on quitting. Thirty-five quit using NRT and thirty quit without NRT. Biomarkers of inflammation, endothelial function, oxidative stress, and lipids were quantified at baseline when actively smoking and after cessation in the presence of NRT or not.

Results: Our previous study showed major changes in biomarkers on quitting compared to the smoking state. They reflected a decrease of inflammation, endothelial activation, and oxidative stress, as well as an improved lipid profile. However, these changes did not depend on the treatment since no difference was found when comparing NRT users and non-users.

Conclusion: These results may indicate that NRT has no effect on inflammation, endothelial function, oxidative stress, and lipids, when using as a medication aid for quitting smoking.

4.1.2 Introduction

Tobacco use is a leading cause of premature mortality worldwide and is responsible for 8 million deaths each year [4]. Smoking is associated with many health problems, including cardiovascular diseases (CVDs), cancers, diabetes and respiratory diseases [5]. The risk of disease development is reduced on quitting, therefore, it is a public health priority to encourage smoking abstinence [6,7]. However, cigarette smoking is an addiction disorder (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) [8]); successful cessation rate without any support is only 4% [9].

Many smoking cessation methods exist to help smokers quit. The combination of behavioural support and pharmacological treatments seems to be the most effective method [9–11]. Among treatments, nicotine replacement therapy (NRT) is widely used and increases rates of quitting by 50–70%. Nicotine replacement products (NRPs) such as gum, tablet, nasal or oral spray, inhaler, or transdermal patch contain pure nicotine, the major alkaloid responsible for tobacco addiction. Nicotine replacement allows reducing the severity and the duration of withdrawal symptoms [3,10].

While cigarette smoking is known to lead to endothelial dysfunction, oxidative stress, inflammation, and altered lipid metabolism, the role of nicotine alone in these mechanisms remains still unclear and largely controversial [12–14]. For example, during the coronavirus disease (COVID-19) pandemic, some authors raised that nicotine and consequently tobacco may inhibit inflammatory processes and in this way may have a protective action concerning COVID-19 [15,16]. Studies *in vitro* or in experimental animals showed that nicotine increased bioavailability of the vasoconstrictor endothelin-1 (ET-1) and inhibited endothelial production of two vasodilators – nitric oxide (NO) and prostacyclin (PGI_2). These alterations in endothelial function is associated with oxidative stress, for instance, reactive oxygen species (ROS) are produced by the uncoupled endothelial NO synthase (eNOS) [14,17]. This mechanism promotes an inflammatory state by the expression of chemokines and cytokines due to a ROS-damaged endothelium becoming permeable to cells supposed to remain in blood [17–19]. However, these observations are in contradiction with studies indicating an anti-inflammatory effect of nicotine through its binding to the α7 nicotinic acetylcholine receptor (nAChR), which is related to the immune response. Nicotine has been shown to inhibit or down-regulate cytokines production in animal studies [20,21]. Some human studies also suggested the use of nicotine as a therapeutic agent in several diseases and disorders (Parkinson's disease, ulcerative colitis, …) [15,16,20,21]. The mechanism involving nicotine as a factor in lipid dysmetabolism is also poorly understood. An animal study showed that nicotine exposure led to enhancing xanthine oxidase (XO) activity, resulting in increased triglycerides, triglycerides/high-density lipoproteins (HDL) ratio, and lipid peroxidation [22].

The above described negative effects of nicotine on inflammation, oxidative stress, endothelial function and lipid metabolism may prevent prescribers to provide NRT for smoking cessation. Therefore, it seemed to be of interest to determine the effects of NRT on biomarkers reflecting these functions. This study aimed to compare the changes in biomarkers of the endothelial function, oxidative stress, inflammation, and lipids, in smokers who successfully quit smoking and who were or were not exposed to NRT.

4.1.3 Materials and Methods

Subjects

This study follows on from a previous one addressing the concentration changes of endothelial dysfunction-related biomarkers on quitting [Colsoul et al., 2022*a*]. Participants are the same. Sixty-five cigarette smokers, 33 men and 32 women aged 26 to 80 years, were recruited through the tobaccology unit of the Centre Hospitalier Universitaire (CHU) of Catholic University of Louvain (UCL) (Namur, Belgium). All were in good health, without any acute pathology. They were followed during their cessation program: thirty-five used Nicotine replacement therapy (NRT) products (gum, transdermal patches, oral sprays, or inhalers), 22 resorted to varenicline (Champix®), and 8 quit without medication (30 NRT non-users). These data are based on the medical prescription and the compliance was assessed from self-reports during the tobacco consultation. Participants in this study provided informed consent. The project was approved by the CHU UCL Namur's ethics committee and was led according to the Declaration of Helsinki and the Good Clinical Practice guidelines [23].

Clinical Protocol

Urine and blood samples from smokers were collected to analyse biological parameters of interest. Urine, one aliquot of heparin whole blood, and one aliquot of EDTA whole blood were immediately frozen at -20°C for later batch analyses. Blood tubes were then centrifuged (15 minutes, 5±3°C, 5950 g, SL16R, Thermo Scientific, USA) to collect serum and heparinised plasma. Some analyses were directly performed on fresh material while

aliquots of serum and plasma were frozen at -20°C for batch analyses – chromatographic assays and enzyme linked immunosorbent assays (ELISA). This protocol was performed twice for all patients: when actively smoking and during their smoking cessation program with or without medication. Participants had to quit smoking for at least 21 days for this second visit. Self-reported tobacco abstinence was confirmed by the analysis of exhaled carbon monoxide (CO) and urinary anatabine and anabasine [24,25].

Quantitative analyses instruments

Some parameters were measured directly after blood collection and centrifugation using VITROS® 5600 System and VITROS® 3600 System from Ortho Clinical Diagnostics (Raritan, USA).

The chromatographic systems were products of Waters (Milford, USA): a high-performance liquid chromatography (HPLC) system connected to an ultraviolet (UV) detector (Alliance 2695, detector 2489, Waters) and an ultra-high performance liquid chromatography (UHPLC) system (Acquity UPLC® H-Class). Depending on the analysis, the latter was coupled with photodiode array detector (Acquity PDA), fluorescence detector (Acquity FLR), or mass detector (Acquity QDa). Samples (urine, plasma, or serum) were divided into batches and defrosted the day of analysis. Systems were calibrated each day of analysis. Data were acquired and processed by Empower 3 (Waters).

Washing steps of ELISA were performed by a microplate strip washer (ELx50, Agilent, Santa Clara, USA) and readings by an absorbance microplate reader (800TS, Agilent). ELISA were carried out on defrosted serum aliquots, allowing batch analyses.

Batch analyses from serum aliquots were also performed on BN ProSpec® System (Siemens Healthcare, Saint-Denis Cedex, France).

Biological parameters evaluation

Smoking status

Self-reported abstinence was confirmed by the exhaled carbon monoxide (CO) measurement (Smokerlyzer® monitor, Bedfont Scientific Ltd, Harrietsham, UK). A threshold of 5 ppm was considered compatible with smoking abstinence [25]. Free nicotine, cotinine, *trans*-3'-hydroxycotinine (3HC), anatabine and anabasine were quantified in urine by UHPLC connected to a mass detector by a previously described method [26].

Oxidative stress

Uric acid and iron concentrations in serum were determined by colorimetry on VITROS 5600. Ferritin was measured in serum by immunoassay on VITROS 3600. Transferrin was quantified in serum by turbidimetry (reagent pack TFTUR-C00, DiAgam, Ghislenghien, Belgium) on VITROS 5600.

Glutathione peroxidase (GPX) was quantified in defrosted heparin whole blood aliquot by an enzymatic method (kit RANSEL, Randox, Crumlin, UK). Superoxide dismutase (SOD) level was determined in defrosted EDTA whole blood aliquot using a colorimetric activity kit (kit RANSOD, Randox, Crumlin, UK). Both were performed in batch on VITROS 5600.

Chromsystems kits (Munich, Germany) were used to quantify vitamine C in plasma and coenzyme Q10 and β-carotene in serum. Detection was performed by HPLC coupled with UV detector. Vitamine A, vitamine E, and malondialdehyde were extracted from plasma using corresponding Chromsystems kits. Vitamines A and E detection was then achieved through an UHPLC reverse phase column (Acquity UPLC HSS T3 1.8 µm 2.1 X 50 mm, Waters) with a flow rate of 0.6 ml/min and photodiode array detection (scan 210-400 nm). Malondialdehyde was quantified using an UHPLC reverse phase column (Luna Omega 1,6 µm C18 100Å 100X2,1 mm, Phenomenex, Torrance, USA) with a flow rate of 0.3 ml/min and fluorescence detection (excitation wavelength: 515 nm; emission wavelength: 553 nm).

Anti-oxidized LDL (oxLDL) antibodies in serum could be assessed by ELISA kit (Biomedica Medizinprodukte GmbH, Wien, Austria).

Inflammatory status

ELISA kits from Abcam B.V. (Amsterdam, Netherlands) allowed to measure monocyte chemoattractant protein 1 (MCP-1) and interleukines (IL) 6, 8, and 10 in serum. Tumor Necrosis Factor (TNF)-α was assessed in serum by an ELISA kit from Tecan (IBL International GmbH, Hamburg, Germany). High-sensitivity C-reactive protein (hs-CRP) was quantified in serum by nephelometry using the BN ProSpec.

Endothelial function

All parameters for the estimation of the endothelial function in this study – Asymmetric dimethylarginine (ADMA), soluble vascular cell adhesion molecule (sVCAM), and soluble intercellular adhesion molecule (sICAM) – were measured in serum by ELISA kits (MT-Diagnostics B.V., Etten-Leur, Netherlands).

Lipid profile

Serum cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides were assessed by enzymatic methods on VITROS 5600. BN ProSpec was used to quantify lipoprotein (a) (Lp(a)), apolipoprotein A1 (ApoA1), and apolipoprotein B (ApoB) by nephelometry. Small dense LDL cholesterol was evaluated with an ELISA kit (Cubasio, Houston, USA).

Statistical analyses

Due to the lack of normality of the data assessed by Shapiro-Wilk test, results are expressed by median with interquartile range into bracket. The comparison of the patients' smoking status when actively smoking and during their smoking cessation program was performed by Wilcoxon signed-rank test. The comparison of the biomarkers modifications when quitting with or without NRT was performed by Wilcoxon Rank Sum Test. Differences were

considered significant when P<0.05. Analyses were carried out using MedCalc® (version 20.109, MedCalc Software Ltd, Ostend, Belgium).

4.1.4 Results

Cigarette abstinence verification

The patients' smoking status was established from the cigarettes consumption self-report, the carbon monoxide (CO) measurement, and the quantification of specific tobacco biomarkers in urine. For each patient, these data were collected at baseline when actively smoking and after smoking cessation (Table 1). The time of abstinence was variable depending on the patient (median: 70 days, interquartile range: 44.5-104.5 days) but was at least 21 days. Parameters decreased significantly when smoking was stopped. Table 1 shows that nicotine and its metabolites (cotinine and 3HC) were still observed after smoking cessation, since a part of patients received nicotine replacement therapy (NRT). Absence of cigarette smoking was proved by the low levels of anatabine and anabasine, two alkaloids not derived metabolically from nicotine.

Table 1: Patients' smoking status at baseline (active smoking) and after minimum 21 days of abstinence (smoking cessation).

	Active smoking Median (Interquartile range) (n=54)	**Smoking cessation** Median (Interquartile range) (n=54)	**P**
No. of cigarettes per day*	20 (10 – 25)	0 (0 – 0)	<0.0001
CO (ppm)	17.5 (13.0 – 25.0)	2.0 (1.0 – 2.0)	<0.0001
Nicotine (µg/ml)	0.612 (0.267 – 1.006)	0.055 (0.055 – 0.235)	<0.0001
Cotinine (µg/ml)*	1.159 (0.831 – 1.634)	0.146 (0.010 – 0.880)	<0.0001
3HC (µg/ml)	3.506 (1.651 – 6.029)	0.413 (0.020 – 2.271)	<0.0001
Anatabine (ng/ml)	5.803 (2.206 – 11.057)	0.029 (0.029 – 0.071)	<0.0001
Anabasine (ng/ml)	1.866 (0.582 – 8.062)	0.032 (0.029 – 0.198)	<0.0001

CO, carbon monoxide; 3HC, *trans*-3'-hydroxycotinine. *n=64

Changes in tobacco biomarkers concentration on quitting with and without nicotine replacement

The decreases of nicotine, cotinine and 3HC levels on quitting were significantly lower for patients using nicotine replacement compared to those not using (Table 2). No significant difference was observed in anatabine and anabasine decrease when quitting with NRT or without.

Table 2: Tobacco biomarkers modification when stopping smoking with or without nicotine replacement therapy.

	Changes in NRT users Median (Interquartile range) (n=30)	**Changes in non-users** Median (Interquartile range) (n=24)	**P**
Nicotine (µg/ml)	-0.189 (-0.563 – 0.044)	-0.531 (-1.033 – -0.179)	0.0125
Cotinine (µg/ml)	-0.209 (-0.872 – 0.164)	-1.107 (-1.623 – -0.831)	<0.0001
3HC (µg/ml)	-0.560 (-3.810 – 0.847)	-4.098 (-5.825 – -2.094)	0.0005
Anatabine (ng/ml)	-5.123 (-9.385 – -1.499)	-6.102 (-12.042 – -2.653)	0.1858
Anabasine (ng/ml)	-0.947 (-6.591 – -0.274)	-2.278 (-8.526 – -0.682)	0.1055

3HC, *trans*-3'-hydroxycotinine

Effect of the nicotine replacement therapy on biomarkers of inflammation, endothelial function, oxidative stress, and lipid profile on quitting

Changes in biomarkers of inflammation, endothelial function, oxidative stress, and lipid profile when smoking was stopped are shown in Tables 3. No significant difference was observed when comparing groups using or not using NRT.

Table 3a: Concentration changes in inflammation biomarkers on quitting smoking with or without nicotine replacement therapy.

	Changes in NRT users Median (Interquartile range) (n=35)	**Changes in non-users** Median (Interquartile range) (n=30)	**P**
IL-6 (pg/ml)	-0.3 (-1.4 – 0.9)	-0.7 (-1.5 – 0.3)	0.1174
IL-8 (pg/ml)	-0.9 (-3.1 – 2.2)	0.5 (-1.4 – 1.7)	0.2925
IL-10 (pg/ml)	0.0 (-0.3 – 0.9)	0.0 (-0.2 – 0.2)	0.5540
TNF-α (pg/ml)	0.0 (-0.1 – 0.0)	0.0 (-0.1 – 0.0)	0.9101
MCP-1 (pg/ml)	-0.7 (-30.7 – 25.2)	0.4 (-51.8 – 11.2)	0.5895
hsCRP (mg/l)	-0.16 (-1.04 – 0.43)	-0.14 (-1.23 – 0.27)	0.6170

IL, interleukine; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; hsCRP, high-sensitivity C-reactive protein.

Table 3b: Concentration changes in endothelial function biomarkers on quitting smoking with or without nicotine replacement therapy.

	Changes in NRT users Median (Interquartile range) (n=35)	**Changes in non-users** Median (Interquartile range) (n=30)	**P**
ADMA (µmol/l)	0.06 (-0.05 – 0.14)	0.02 (-0.13 – 0.12)	0.3367
sVCAM (ng/ml)	-7.0 (-121.9 – 60.7)	12.2 (-71.1 – 96.1)	0.2415
sICAM (ng/ml)	-28.0 (-78.3 – -1.0)	-10.5 (-68.5 – 49.0)	0.0947

ADMA, asymmetric dimethylarginine; sVCAM, soluble vascular cell adhesion molecule; sICAM, soluble intercellular adhesion molecule.

Table 3c: Concentration changes in oxidative stress biomarkers on quitting smoking with or without nicotine replacement therapy.

	Changes in NRT users Median (Interquartile range) (n=35)	**Changes in non-users** Median (Interquartile range) (n=30)	**P**
SOD (U/ml)	-3.0 (-21.4 – 28.4)	-17.0 (-61.7 – 14.2)	0.2500
GPX (U/l)	599 (-3038 – 2680) (n=18)	-1886 (-2858 – 427) (n=15)	0.1481
MDA (µg/l)	0.68 (-3.21 – 2.02) (n=19)	-1.09 (-3.70 – -0.01) (n=17)	0.1406
Uric acid (mg/dl)	0.42 (0.01 – 0.78)	0.31 (-0.26 – 0.62)	0.3117
Fer (µg/dl)	3.0 (-25.3 – 25.8)	16.0 (-21.3 – 36.8)	0.4420
Ferritin (ng/ml)	0.5 (-18.0 – 11.8)	-1.2 (-10.3 – 14.0)	0.7978
Transferrin (g/l)	-0.1 (-0.2 – 0.1) (n=23)	0.0 (-0.1 – 0.2) (n=16)	0.4075
Vitamin A (mg/l)	0.04 (-0.08 – 0.12)	-0.01 (-0.04 – 0.07)	0.2925
Vitamin E (mg/l)	-0.60 (-2.86 – 2.83)	-0.55 (-3.20 – 1.65)	0.7322
Vitamin C (mg/l)	0.92 (-2.33 – 3.47)	0.46 (-1.30 – 4.34)	0.7353
Coenzyme Q10 (µg/l)	-19.84 (-206.15 – 94.28)	-31.08 (-140.26 – 89.37)	0.6170
β-carotene (ng/ml)	-7.18 (-41.34 – 37.64)	7.47 (-23.67 – 31.14)	0.3237
Anti-oxLDL (U/l)	4.68 (-37.10 – 79.70)	3.55 (-30.30 – 57.00)	0.9036

SOD, superoxide dismutase; GPX, glutathione peroxidase; MDA, malondialdehyde; anti-oxLDL, anti-oxidized LDL antibodies.

Table 3d: Concentration changes in lipids on quitting smoking with or without nicotine replacement therapy.

	Changes in NRT users Median (Interquartile range) (n=35)	**Changes in non-users** Median (Interquartile range) (n=30)	**P**
Cholesterol (mg/dl)	-3.0 (-12.3 – 14.5)	-6.5 (-18.0 – 12.0)	0.4491
HDL (mg/dl)	3.0 (-3.5 – 6.8)	5.0 (-7.0 – 12.0)	0.5187
LDL (mg/dl)	-8.4 (-17.4 – 1.3)	-8.0 (-21.8 – 6.3)	0.7521
Triglycerides (mg/dl)	-6.0 (-31.0 – 38.3)	2.0 (-41.0 – 47.0)	0.7571
Lp(a) (g/l)	0.09 (0.04 – 0.31)	0.08 (0.03 – 0.25)	0.3335
ApoA1 (g/l)	0.06 (-0.03 – 0.14)	0.05 (-0.06 – 0.33)	0.6309
ApoB (g/l)	-0.06 (-0.11 – 0.03)	-0.04 (-0.13 – 0.05)	0.9371
sdLDL (nmol/ml)	-62 (-214 – 173)	-163 (-495 – 145)	0.2581

HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lp(a), lipoprotein (a); Apo, apolipoprotein; sdLDL, small dense LDL.

4.1.5 Discussion

The subjects smoking status was assessed based on self-report and the quantification of tobacco biomarkers. The active smoking status at baseline was confirmed by the exhaled CO level above the reported cutpoint (5 ppm [25]) to distinguish smokers and non-smokers. Urinary cotinine concentration was also above the cutpoint of 0.03-0.05 µg/ml [25]. Active tobacco users are reported to have urinary concentrations >0.1 µg/ml for nicotine, >0.4 µg/ml for 3HC [24,27,28], >0.097 ng/ml for anatabine, and >0.236 ng/ml for anabasine [Colsoul et al., 2022*b*]. Cigarette smoking abstinence was then evaluated by the same markers. CO measurement confirmed the cessation of combustible tobacco, the median and the interquartile range being <5 ppm. Nicotine and its derived metabolites (cotinine and 3HC) were still found in urine (not below the cutpoint values) given the use of NRT for a part of the subjects. However, anatabine and anabasine, not derived metabolically from

nicotine, were below the cutpoints. These alkaloids are not found in users of pharmaceutical grade nicotine, contrary to commercial alternative nicotine products users [25,29].

As expected, the decreases observed in nicotine, cotinine, and 3HC levels on quitting were lower when using NRT compared to not using, due to the partial nicotine replacement. Since NRT products do not contain any anatabine and anabasine [25], no significant difference were found in the decrease of these biomarkers when comparing smoking cessation with or without NRT.

Changes in the concentration of biomarkers reflecting inflammation, endothelial function, oxidative stress, and lipid profile on quitting were observed. Our previous study has shown these changes by the follow-up of smokers who entered a smoking cessation program [Colsoul et al., 2022*a*]. The biomarkers were quantified at baseline and post-quit, which demonstrated a decrease of inflammation, endothelial activation, and oxidative stress on quitting. An improvement of the lipid profile was also observed. However, these reported changes did not appear to be influenced by NRT exposure. Actually, no significant difference was observed when comparing concentration changes in biomarkers of inflammation, endothelial function, oxidative stress, and lipid profile, in NRT users or non-users.

To date, there is a lack of data addressing the nicotine exposure effect in humans in real conditions of nicotine use for quitting. Studies are performed *in vitro* or in animal models and results are sometimes contradictory. Some studies reported that nicotine led to endothelial dysfunction by the deregulation of vasoconstrictors and vasodilatators biodisponibility, which was associated with oxidative stress and inflammation triggers [17–19]. These data could curb doctors to prescribe NRT for quitting while it is known that it increases the cessation success rate [3]. Conversely, other studies claimed an anti-inflammatory effect of nicotine through the cholinergic anti-inflammatory pathway, inducing the suppression of proinflammatory cytokines production [20,21]. This effect of nicotine could protect against infection by different virus, including influenza A, SARS-CoV-2, etc. [15,20]. Authors mention a therapeutic potential of nicotine [20,21]. In this study, no difference in terms of cytokines production was observed when using or not using nicotine

with an abstinence of at least 21 days. Similarly, nicotine did not seem to lead to endothelial dysfunction and oxidative stress on quitting. An animal study reported an increase of triglycerides, triglycerides/HDL ratio, and lipid peroxidation due to nicotine exposure [22] but this study does not confirm this hypothesis in humans in cessation.

A limitation of this study is the non-standardisation of the duration of cigarette abstinence and NRT use (median: 70 days, interquartile range: 44,5-104,5 days). An hypothesis to explain that no effect of nicotine is observed in biomarkers when stopping is that the use of nicotine could be too short (at least 21 days) to be able to detect any effect. However, this study was performed in real conditions with smokers enrolled in a cessation program and samples were collected before a possible relapse. The data obtained here can inform doctors about the short-term use of NRT. Another limitation of this project is the fact that it relates only to cigarettes smokers on quitting and not to users of other forms of tobacco. The nicotine replacement was also evaluated for admitted NRT products only. Alternative nicotine products were not addressed. Finally, these first results should be confirmed in a larger population.

4.1.6 Conclusion

It is admitted that cigarette smoking leads to endothelial dysfunction, oxidative stress, inflammation, and altered lipid metabolism, but the effect of exposure to nicotine alone is not yet established. This is especially of interest since NRT, containing only nicotine and not the other cigarette constituents, are useful for achieving successful tobacco cessation. Biomarkers of the endothelial function, oxidative stress, inflammation, and lipids, were quantified at baseline and when stopping smoking with or without NRT. Changes in these biomarkers were observed on quitting, but these changes were not significantly different when using or not using NRT. Therefore, NRT products do not seem to affect positively or negatively the studied functions. These data seem to support the safety profile of the NRT products. It could constitute an additional argument for doctors to prescribe NRT to smokers enrolled in a cessation program in order to increase their chances of success.

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CONCLUSION AND PERSPECTIVES

Smoking is the most preventable cause of early death worldwide. It is associated with many pathological conditions and it is, especially, a primary risk factor for cardiovascular diseases (CVDs). The mechanisms by which cigarette smoking induces and promotes CVDs are complex and not clearly identified. Nevertheless, endothelial dysfunction, inflammation, oxidative stress, and altered lipid metabolism are reported as key pathways and are all interconnected.

It is of interest to conduct new studies in order to improve the understanding of the implied mechanisms in CVDs development. Moreover, it is also crucial to provide new evidence about the potential involvement of nicotine, the major tobacco alkaloid responsible for cigarette addiction. Actually, the role of this cigarette smoke constituents in CVDs development is still unclear and largely controversial in the literature. The elucidation could allow taking a stance concerning non-tobacco nicotine delivery products, such as electronic cigarette or nicotine containing medications for smoking cessation.

In this project, we studied the endothelial dysfunction, the first step in CVDs development, in smokers and nicotine users through the quantification of related biomarkers. Comparisons with non-smokers were performed. The study was divided into four chapters reporting the experimental work.

In the first chapter, we developed new quantification methods to measure biomarkers concentration in participants' body fluids. The first development was performed to quantify cotinine in urine. It is the biomarker of choice to reflect the nicotine uptake. Our new method resorts to ultra-high performance liquid chromatography (UHPLC) connected with a mass detector, which is less expensive than a mass spectrometer while benefiting from the specificity of the mass. We used the same strategy to develop a second method to measure five tobacco alkaloids simultaneously. With this method, we were able to discriminate between tobacco users, nicotine users, and non-users. The third and the last development concerned the quantification of malondialdehyde (MDA), a biomarker of oxidative stress. We used an UHPLC system with fluorescence detector. All the developed method were validated according to ISO15189 standard.

In the second chapter, we characterised smoking-induced endothelial dysfunction by the comparison of related biomarkers concentrations in smokers and non-smokers. We showed that inflammation seemed increased in smokers by the increased levels of interleukine-8 and C-reactive protein (CRP). Endothelial activation in smokers could be visible through the increase of the intercellular and vascular adhesion molecules (ICAM and VCAM). Oxidative stress could be observed increased in smokers given that vitamin C, β-carotene, and uric acid, three antioxidants, were found at lower concentrations and MDA at higher concentration. Lipid metabolism seemed also affected: for example, triglycerides level was higher in smokers. In this chapter, we also reported the possibility of assessing individuals' tobacco status by using biomarkers other than nicotine and its metabolites. It is particularly of interest to differentiate nicotine users, such as in nicotine replacement therapy (NRT), from tobacco smokers. In this context, we evaluated the discriminating features of endothelial dysfunction-related biomarkers, but also of two minor tobacco alkaloids (anatabine and anabasine). We showed that the latters allowed to achieve good diagnosis with high sensitivity and specificity while they were put down in the literature due to poor cutoffs. We also found two different combinations of several biomarkers (tobacco biomarkers excluded) allowing to classify correctly >84% of cases. It opens the prospect of assessing patients' tobacco use based on classic parameters of blood test, without the need of specific equipment for medical laboratories.

In the third chapter, we investigated if endothelial dysfunction observed in smokers could be reversible on quitting. The follow-up of smokers in cessation and the measurement of the biomarkers concentration changes provided evidence that smoking cessation could allow to improve endothelial function. The level of CRP tended to decrease and the concentration of interleukine-6 was also lower on quitting, reflecting a decrease of inflammation. The level of ICAM on quitting tended toward the one observed in non-smokers. The same was found for vitamin C and uric acid. Concentration of high-density lipoprotein (HDL) was higher on quitting and concentration of low-density lipoprotein (LDL) was lower, despite the weight gain observed with the cessation. All these observations show

that smokers who quit could improve their endothelial function and reduce their risk for CVDs.

In the fourth chapter, we compared the biomarkers concentration changes on quitting for abstinents using NRT or not. Since the role of nicotine in endothelial dysfunction and CVDs is still unclear, we wanted to address the effect of NRT on biomarkers. We found that changes observed in biomarkers concentrations on quitting did not depend on the treatment, with or without nicotine. This observation suggests that nicotine does not influence positively or negatively the endothelial function and, therefore, the risk for CVDs.

In conclusion, this project contributes to the understanding of endothelial dysfunction observed in smokers. We confirmed that endothelial dysfunction in smokers seemed associated with increased inflammation and oxidative stress as well as altered lipid profile. Specific biomarkers of these pathways were found impacted by cigarette smoking. We showed that these adverse effects on endothelial function could be reversible on quitting. Finally, we provided evidence that nicotine for quitting does not affect the endothelial function.

This work present some limitations that we can address here. This study is based on the comparison of a large panel of biomarkers in different groups of individuals in order to identify the effect of tobacco and NRT in endothelial dysfunction. This large panel of biomarkers leads to multiple statistical comparisons. However, all selected variables were essential to reflect the different mechanisms involved in endothelial activation, inflammation, oxidative stress, and dyslipidaemia. For this reason, some variables could be found affected by the smoking status while others of the same category were not. We analysed the effect of smoking cessation in endothelial dysfunction, but some participants resorted to a treatment that could influence the observed changes in biomarkers concentration. The ideal would have been to compare biomarkers in smokers at baseline and post-quit without any treatment. Unfortunately, this study was performed in real conditions of smoking cessation program and quitting without help is difficult. Therefore, few smokers who quit without treatment could be followed. However, the influence of the

treatment was considered by comparing biomarkers concentration on quitting in NRT users and non-users. The latters quit without medication or with varenicline. Another limitation is the use of varenicline for some participants because there is similarity between varenicline and NRT in the mechanism of action (agonist at nicotinic acetylcholine receptors (nAChRs)). Finally, some effects could not have been detected due to the small sample size.

The perspectives of this work are: (i) the validation of the two models to discriminate between smokers and non-smokers without the use of tobacco biomarkers. Another population has to be recruited to test the discrimination capacity of our models. A cost-effectiveness study could also be carried out; (ii) the evaluation of the endothelial dysfunction-related biomarkers in former smokers after they have stopped their treatment for cessation. It could allow to assess the reversibility of endothelial dysfunction without any influence of treatment; (iii) the same study could be performed on a larger cohort to confirm our results and potentially detect additional changes in biomarkers concentrations.

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